Prion infectivity is closely related to neurological disorders called TSE which include human diseases such as Creutzfeldt–Jakob disease (CJD), Fatal Familial Insomnia and Gerstmann Straüssler Scheinker syndrome.

CJD is classified as sporadic, genetic, iatrogenic including the CJD variant associated with bovine spongiform encephalopathy. The infectious agent responsible for this disease was called a "prion" by Prusiner [1]. It is a glycoprotein which is normally present in the physiological form (PrP^c) which becomes pathological in CJD (PrP^{sc}). The transition process from the physiological to the pathological form is complex [2]. Scientists have demonstrated a trans-conformational change between both protein forms [3]. PrP^c primarily contains α helix, though PrP^{sc} contains more β sheets in its three-dimensional structure [4]. CJD is characterized by intra-cerebral accumulation of abnormal prion protein which is partially protease resistant (PrP^{res}). Cleavage of PrP^{sc} by proteinase K results in two types of PrP^{res} after western blot, type 1 and type 2, according to Parchi's classification [5].

Studies on the elimination of viral contamination from biopharmaceutical products (which are prepared from human cells) have been conducted using nanofiltration [6–8]. This nanofiltration method has been effective on many viruses. Albumin solution safety nanofiltration has been conducted on scrapie prion protein [9].

The purpose of this research is to study the efficacy of nanofiltration on CJD PrP^{sc} in order to introduce this method into the manufacturing process of a therapeutic immunoglobulin solution Lymphoglobuline®.

Lymphoglobuline® equine anti-human thymocyte immunoglobulin is a selective immunosuppressive agent acting mainly on human T lymphocytes. It recognizes most of the molecules involved in the cascade of T-cell activation during graft rejection, such as T-cell receptor and CD3, HLA class 1 molecules, CD4 and CD8 co-receptors, co-activation molecules or adhesion molecules CD2, CD5, and CD18. The therapeutic indications are the followings:

- immunosuppression for transplantation: prevention and treatment of graft rejection;
- treatment of aplastic anemia.

During the process of purification of the equine anti-human thymocytes a step of nanofiltration was added for the viral security.

2. Materials and methods

2.1. Biopharmaceutical product

Lymphoglobuline® is an anti-thymocyte equine immunoglobulin that induces immunosuppression as a result of T-cell depletion and immune modulation. It is approved for the prevention and treatment of rejection episodes in kidney, pancreas or liver transplantation. In hematology, Lymphoglobuline® is approved for treatment of aplastic anemia and in the treatment of steroid resistant graft versus host disease.

In the Lymphoglobuline® manufacturing process, human thymocytes, membrane red blood cells and placenta are used. These human elements represent a virtual potential source of contamination of Lymphoglobuline®.

2.2. Human source of pathological prion protein

After the histological, immunohistochemical and biochemical analyses of post-mortem human brains, one case of definite CJD, and one non-CJD were chosen. The anatomic site chosen was the frontal cortex. The CJD case selected was characterized by the presence of PrP^{res} type 1 in western blot analysis according to Parchi's classification and by synaptic deposits of PrP^{sc} with an immunohistochemical technique. The same human cortex was used as source of PrP^{sc} for the reference scale and for the nanofiltration samples.

2.3. Sample preparation

2.3.1. Human brain homogenate

Frontal cortex of CJD and non-CJD cases was spiked in PBS buffer, 1:10 at final dilution. These homogenates were filtered successively with needles of 0.6 mm and 0.5 mm diameter in order to obtain homogenous preparation. After centrifugation at 1000g for 5 min, supernatants were applied to nanofiltration process.

2.3.2. Reference scale

This reference scale was prepared with series of dilutions of CJD brain homogenate in Lymphoglobuline® from 1:10 to 1:20,000. This reference scale was based on the technique used by Lee et al. [10,11].

2.3.3. Nanofiltration samples

These samples were prepared using CJD brain homogenate dilutions in Lymphoglobuline®. Three different samples were produced; samples at a high PrP^{sc} dilution (1:500), samples at a moderate PrP^{sc} dilution (1:100) and samples at a low PrP^{sc} dilution (1:10). Each dilution was prepared for three samples, one non-nanofiltrated (control) and two nanofiltrated. These samples were prepared as a reference scale with an adaptation of the method used by Lee et al. [11].

2.4. Filtration

Small-sized (membrane diameter: 47 mm) Pall® filters (hydrophilic Polyvinylidene fluoride microporous membrane) with mean pore sizes for Pall® DVD of about 0.1 μm, Pall® DV50 of about 50 nm and Pall® DV20 of about 20 nm were used successively in the nanofiltration process. The filtration mode was conducted at a constant membrane pressure of 3 bars. The samples underwent nanofiltration in the following order: negative control, CJD samples at a high PrP^{sc} dilution (1:500), CJD samples at a moderate PrP^{sc} dilution (1:100), CJD samples at a low PrP^{sc} dilution (1:10) and negative control (Fig. 1).

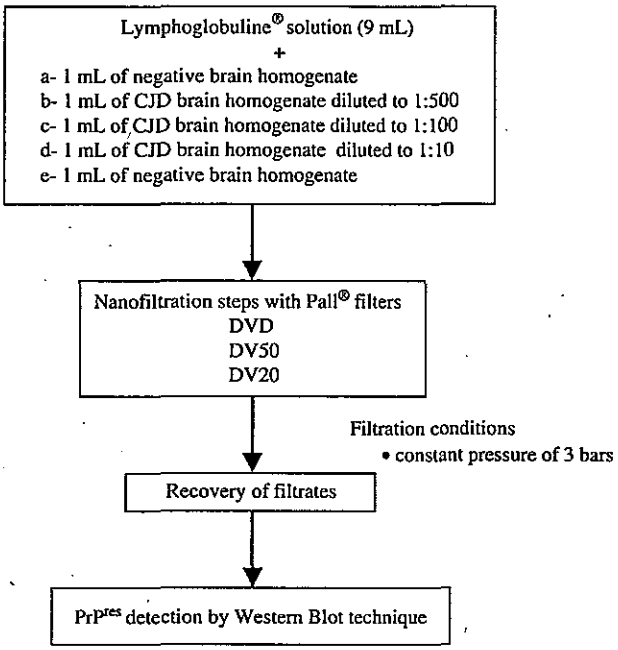

Fig. 1. Nanofiltration process. (a) Negative control sample at 1:10 in Lymphoglobuline®; (b) CJD sample at a high PrP^{sc} dilution (1:500) in Lymphoglobuline®; (c) CJD sample at a moderate PrP^{sc} dilution (1:100) in Lymphoglobuline®; (d) CJD sample at a low PrP^{sc} dilution (1:10) in Lymphoglobuline®; and (e) negative control sample at 1:10 in Lymphoglobuline®, ×2, produced in duplicate.

The nanofiltration material was treated with sodium hydroxide (2 M) for 1 h between each nanofiltration of different PrP^{sc} dilution samples.

2.5. PrP^{res} detection

The western blot technique was used to detect PrP^{res} after proteinase K treatment [12]. The anti-prion protein antibody revealed three strips of a molecular weight between 30 and 22 kDa (Fig. 2) corresponding to the biglycosylated, monoglycosylated and unglycosylated forms. Then, PrP^{res} was revealed by chemiluminescence. This technique was used to detect PrP^{res} in reference scale samples and in samples before and after nanofiltration.

The reference scale samples and samples for nanofiltration were produced and developed by the western blot technique under the same conditions and in the same time.

2.6. Determination of reduction factors

The reduction factors defined as the reduced titer versus the real titer present in the spiked sample were determined by comparing the PrP^{res} signal of samples before and after nanofiltration with the PrP^{res} signal of reference scale. After this comparison, we determined a reduction factor (log) for each sample.

3. Results

The reference scale ranges from 1:10 to 1:20,000 dilutions of CJD brain homogenates. From the 1:10 to 1:2000 dilutions,

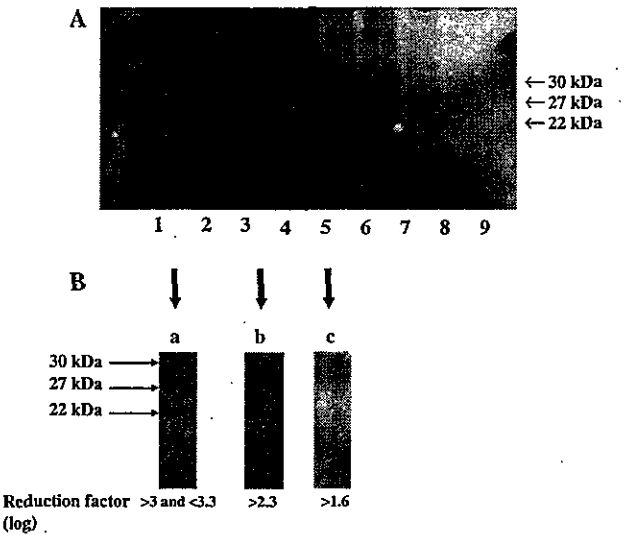

Fig. 2. (A) Reference scale. (1) PrP^{sc} sample dilution at 1:10 in Lymphoglobuline®; (2) PrP^{sc} sample dilution at 1:20 in Lymphoglobuline®; (3) PrP^{sc} sample dilution at 1:100 in Lymphoglobuline®; (4) PrP^{sc} sample dilution at 1:200 in Lymphoglobuline®; (5) PrP^{sc} sample dilution at 1:1000 in Lymphoglobuline®; (6) PrP^{sc} sample dilution at 1:2000 in Lymphoglobuline®; (7) PrP^{sc} sample dilution at 1:10,000 in Lymphoglobuline®; (8) PrP^{sc} sample dilution at 1:20,000 in Lymphoglobuline®; and (9) negative sample dilution at 1:10 in Lymphoglobuline®. (B) Western blot signal after nanofiltration steps. a. Signal of PrP^{sc} sample dilution at 1:10 after nanofiltration, three strips of PrP^{res} were shown, the reduction factor was between 3 and 3.3 log to the reference scale. b. Signal of PrP^{sc} sample dilution at 1:100 after nanofiltration, strips of PrP^{res} were not detected, the reduction factor was strictly greater than 2.3 log in comparison to the reference scale. c. Signal of PrP^{sc} sample dilution at 1:500 after nanofiltration, strips of PrP^{res} were not detected, the reduction factor was strictly greater than 1.6 log in comparison to the reference scale.

the three strips of PrP^{res} (biglycosylated, monoglycosylated and unglycosylated) were visualized, and for the 1:10,000 and 1:20,000 dilutions, only the monoglycosylated strip was detected. The reference scale was elaborated over a 3.3 log range by the western blot technique (1:10–1:20,000). (Fig. 2A).

The PrP^{res} strips of samples at a low PrP^{sc} dilution (1:10) were detected after nanofiltration with a western blot signal corresponding to the reference scale dilution between 1:10,000 and 1:20,000 (Fig. 2B). The nanofiltration process removed PrP^{sc} by a reduction factor of 3–3.3 log.

The PrP^{res} strips of samples at a moderate PrP^{sc} dilution (1:100) and of samples at a high PrP^{sc} dilution (1:500) were not detected after nanofiltration, these samples after nanofiltration corresponding to reference scale dilutions were greater than 1:20,000 (Fig. 2B). PrP^{sc} was removed by the nanofiltration process with a reduction factor greater than 2.3 log and 1.6 log: the real reduction factors are not available due to limited detection of the western blot assay.

The western blot signal of the negative control sample was negative, no strips of PrP^{res} were detected.

4. Discussion

Pathological prion protein (PrP^{sc}) is highly resistant to inactivation [13] and is not destroyed by known viral inactivation

processes [14]. Effective methods include for example exposure to 1 M sodium hydroxide during autoclaving at 121 °C. This kind of method using chemical agents (sodium hydroxide, chlorine at high concentrations) and physical treatment by autoclaving is very drastic and it is a real problem to inactivate PrP^{sc} in biopharmaceutical products without modifying their therapeutic properties. The reduction of any risk associated with a pharmaceutical product will be dependent on the physical removal of infective material during product manufacture. Many techniques for plasma-derived products, such as ethanol fractionation, depth filtration and chromatographic processes, may contribute to a significant partitioning of prion protein [10,15–18]. Although early applications of nanofiltration targeted viral removal [7,8,19], new data suggest that it may be a specific removal system for prion proteins as well. Human TSE pathogens in diluted brain homogenate were reported to be removed by a Millipore screen-type 0.025 μm membrane filter employed during production of growth hormone [20]. However, only a small quantity of diluted brain homogenate could pass through the membrane. Planova® cartridges with mean pore sizes from 75 to 10 nm were used to filter brain homogenate from mice infected with human TSE [21]. No infectivity was detected in the 35 nm filtrate. The pathogenic agent was estimated to be approximately 40 nm in size. However, some residual infectivity was found in the 10 nm filtrate when 1% Sarkosyl was added to the homogenate [22]. Recently, removal of scrapie agent ME7, a mouse adapted strain of scrapie used as a model for the BSE or vCJD agents by using nanofiltration of a 2% albumin solution spiked with a brain homogenate [23]. The albumin recovery was over 90%. Extent of removal was influenced by the filter type and by the addition of an anionic detergent (Sarkosyl) to the protein solution. An infectivity of 4.93 and 1.61 log was removed using a 35-nm filter without and with detergent, respectively. Moreover, a reduction of infectivity of >5.87 and 4.21 log was obtained using a 15-nm filter in the absence and presence of detergent, respectively. No residual infectivity was detected in any filtrate when using 15 nm or smaller porosity filters. Studies have shown an efficacy of 35–15 nm filters in achieving some removal of prions from biological solutions with the best removal with a 15-nm filter. The data, although encouraging, should be analyzed more accurately due to the tendency of prion spikes to aggregate under the experimental conditions used and with human prion protein because this removal could be dependent on the "strain" of prion protein.

In our study, we wanted to study the efficacy of nanofiltration on human PrP^{sc} in a biopharmaceutical product (Lymphoglobuline®). We used human PrP^{sc} from CJD patients as the contaminant. This contamination condition was important to study the Lymphoglobuline® nanofiltration technique under conditions as close as possible to a possible contamination by human cells used for the preparation of this product. The extent of removal may be influenced by the aggregation, type [24] and conformation of prion proteins and the physicochemical nature of the solution filtered. These parameters were important to choose the PrP^{res} type for the study. Amyloid plaques or focal deposits of PrP^{sc} still remain after homogenizing the cerebral cortex and the hypothesis was made that this kind of PrP^{sc} aggregation could be the result of a bias in the methodology. For this reason, PrP^{res} type 1 associated with synaptic deposits with an immunohistochemical technique was chosen in order to test the Lymphoglobuline® nanofiltration process under worst conditions to test the filters. In this study, Lymphoglobuline® was spiked with brain homogenate at different dilutions (1:10, 1:100, and 1:500). These PrP^{sc} dilutions can be correlated with World Health Organization (WHO) classification of organ infectivity: the low PrP^{sc} dilution corresponding to 1:10 (brain and spinal cord), moderate PrP^{sc} dilution corresponding to 1:100 (spleen, tonsil, lymph node, intestine, placenta...) and high PrP^{sc} dilution corresponding to 1:500 (brain stem, thymus, liver, pancreas, lungs...).

The comparison of the samples before and after nanofiltration showed a reduction factor between 3.3 and 1.6 log in comparison with the reference scale. The reduction factor of samples at a low PrP^{sc} dilution (1:10) was between 3 and 3.3 log. This dilution could correspond to a brain or a spinal cord PrP^{sc} concentration (WHO). The reduction factors for a very high PrP^{sc} concentration obtained illustrate a very good efficacy of the nanofiltration process.

In samples at a moderate PrP^{sc} dilution (1:100) and samples at a high PrP^{sc} dilution (1:500), the PrP^{res} strips were not detected after nanofiltration, the reduction factor was strictly greater than 2.3 and 1.6 log, respectively. The 1:100 dilution could correspond at a spleen or tonsil or lymph node or intestine or placenta PrP^{sc} concentration (WHO) and the 1:500 dilution could correspond to a brain stem or thymus or liver or pancreas or lungs PrP^{sc} concentration (WHO). In conclusion, the data obtained on both these PrP^{sc} dilutions are encouraging because, after nanofiltration, the PrP^{res} signal was not detected, although they are only indicative with probably underestimated reduction factors. Finally, the reduction factor obtained is 3.3 log and seem to demonstrate the efficacy of the nanofiltration process on human CJD PrP^{sc} with a good protein recovery.

Removal may be based on a sieving mechanism or due to adsorption on the membrane. The potential to use nanofiltration as a dedicated step for prion removal may have a significant impact on the safety of biopharmaceutical products and recombinant proteins, when production involves the use of human or animal derived materials, or medicinal products derived from bovine sources [25,26]. This technique has the ability to extend the concept of sterility of biological products from bacteria to, at least, some viruses. Our results suggest that nanofiltration could be also of interest for the removal of human pathological prion proteins.

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ORIGINAL PAPER

Creutzfeldt–Jakob disease and blood transfusion: results of the UK Transfusion Medicine Epidemiological Review study

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Vox Sanguinis

Background and Objectives This paper reports the results to 1 March 2006 of an ongoing UK study, the Transfusion Medicine Epidemiological Review (TMER), by the National CJD Surveillance Unit (NCJDSU) and the UK Blood Services (UKBS) to determine whether there is any evidence that Creutzfeldt–Jakob disease (CJD), including sporadic CJD (sCJD), familial CJD (fCJD), and variant CJD (vCJD) is transmissible via blood transfusion.

Materials and Methods Sporadic CJD and fCJD cases with a history of blood donation or transfusion are notified to UKBS. All vCJD cases aged > 17 years are notified to UKBS on diagnosis. A search for donation records is instigated and the fate of all donations is identified by lookback. For cases with a history of blood transfusion, hospital and UKBS records are searched to identify blood donors. Details of identified recipients and donors are checked against the NCJDSU register to establish if there are any matches.

Results *CJD cases with donation history:* 18/31 vCJD, 3/93 sCJD, and 3/5 fCJD cases reported as blood donors were confirmed to have donated labile components transfused to 66, 20, and 11 recipients respectively. Two vCJD recipients have appeared on the NCJDSU register as confirmed and probable vCJD cases. The latter developed symptoms of vCJD 6.5 years and 7.8 years respectively after receiving non-leucodepleted red blood cells (RBCs) from two different donors who developed clinical symptoms approximately 40 and 21 months after donating. A third recipient, given RBC donated by a further vCJD case approximately 18 months before onset of clinical symptoms, had abnormal prion protein in lymphoid tissue at post-mortem (5-years post-transfusion) but had no clinical symptoms of vCJD. CJD cases with history of transfusion: Hospital records for 7/11 vCJD and 7/52 sCJD cases included a history of transfusion of labile blood components donated by 125 and 24 donors respectively. Two recipients who developed vCJD were linked to donors who had already appeared on the NCJDSU register as vCJD cases (see above). No further links were established.

Conclusion This study has identified three instances of probable transfusion transmission of vCJD infection, including two confirmed clinical cases and one pre- or sub-clinical infection. This study has not provided evidence, to date, of transmission of sCJD or fCJD by blood transfusion, but data on these forms of diseases are limited.

Key words: blood, CJD, familial, sporadic, transfusion variant.

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Introduction

Until 2004, it was generally accepted that Creutzfeldt–Jakob disease (CJD) had not been transmitted by blood transfusion.

Preliminary findings from sheep studies indicate that bovine spongiform encephalopathy (BSE) and scrapie can be transmitted by blood transfusion [1,2]. It is vital to find out whether this also applies to human transmissible spongiform encephalopathies (TSEs) and, in particular, variant CJD (vCJD). The UK is the only country where a significant outbreak of vCJD has occurred and is in a unique position to study this question which has important implications for public health policy. The results reported in this paper are from a study which is being carried out, with ethical approval, to investigate whether or not there is any evidence for the transmission of any type of CJD (sporadic, familial and variant) by blood transfusion.

Materials and methods

CJD surveillance

A surveillance system for CJD, the National CJD Surveillance Unit (NCJDSU), was established in the UK in 1990 with the aim of identifying all cases of CJD in the UK. The methodology of this study has been described previously [3], but in brief involves referral of suspected cases to the Unit from targeted professional groups, including neurologists and neuropathologists; review of suspects by a neurologist from the Unit and review of investigation results and neuropathological material when available. Cases are classified according to standard diagnostic criteria [4,5]. Onset of clinical symptoms for vCJD cases are estimated to the nearest month by NCJDSU on the basis of available clinical information. Details of past medical history, including blood donation or transfusion, are obtained from the family of suspected cases. Following the identification of vCJD in 1996 a collaborative study, the Transfusion Medicine Epidemiological Review (TMER), was established between the NCJDSU and UK Blood Services (UKBS) to search for evidence of transfusion transmission of CJD. The study was granted ethical approval by the local Research Ethics Committee.

Notification of CJD cases with a history of donation

Sporadic CJD (sCJD) and familial CJD (fCJD) cases with a history of blood donation are notified to UKBS retrospectively. For vCJD, all patients who are old enough to have donated blood (> 17 years of age) are notified to UKBS at diagnosis, whether or not there is a known history of blood donation. Upon receipt of notification from the NCJDSU, a search is made for donor records. Current computer databases and archived records (computerized and paper-based records where appropriate) based at individual blood centres are searched using name, date of birth, and previous addresses as identifiers. For CJD cases reported as blood donors, information on dates and places of donation is also used to help locate past donor records. Where donor records are found, all components produced and issued to hospitals are identified and their fate determined, as recorded in hospital blood transfusion laboratory records. Recipient details are then checked against the NCJDSU register to establish if there is a match between these individuals and patients who have developed CJD. Recipients details are also flagged with the Office for National Statistics (ONS) to establish date and certified cause of death.

Plasma used for UK fractionation

Independent of this study, and for regulatory reasons, all plasma derived from donations made prior to a diagnosis of vCJD is notified to UK fractionators so that appropriate actions can be taken.

Notification of CJD cases with a history of transfusion

Information provided by relatives of CJD cases with a previous transfusion history is also passed to UKBS, who then liaise with appropriate hospital blood transfusion laboratories. The laboratories identify whether transfusions took place at the time and place indicated, and, if so, identify the components transfused to the case. Details of donation numbers, component type and date transfused are passed back to the local blood centre and this information is used to identify the donors. Donor details are checked against the NCJDSU register and with ONS. For both donors and recipients, searches before 1980 are now impractical as most hospital records are no longer extant.

Related public health measures

The Department of Health (England) set up a committee (the CJD Incidents Panel) in 2000 to advise health authorities on the management of incidents where patients may have been put at risk of CJD through medical procedures. Cases where a blood donor or blood recipient has later developed vCJD have been referred to the Incidents Panel for consideration and further actions have been recommended and implemented. These further actions are outside the scope of this study and are not reported in this paper.

Results

vCJD cases with history of blood donation

Identification of donors

A total of 150 vCJD cases (out of a total of 160 cases on the NCJDSU register) who were old enough to have been potential blood donors have been notified to UKBS as of 1 March

Table 1 Recipients of blood donated by variant Creutzfeldt–Jakob disease cases by year and blood component transfused ($n = 66$)

Year of transfusion	Blood component transfused	Number of recipients
1980–1984	Whole blood	1
	Red blood cells	1
1985–1989	Red blood cells	2
1990–1994	Red blood cells	9
1995–1999	Whole blood	1
	Red blood cells	15
	Red blood cells – buffy coat depleted[a]	2
	Red blood cells – leucodepleted[b]	2
	Fresh frozen plasma	3
	Cryo-depleted plasma	1
	Cryoprecipitate	1
	Platelets (pooled)	1
2000–2004	Red blood cells – leucodepleted	23
	Fresh frozen plasma – leucodepleted	2
	Platelets (pooled, leucodepleted)	2

[a]Red cells with buffy-coat (containing most of the platelets and white cells) removed by centrifugation and physical separation.
[b]Red cells leucocyte-depleted by pre-storage filtration to $< 5 \times 10^6$/unit according to UK guidelines [6].

2006. Of these, 31 of 150 (21%) were reported to have been blood donors at various times in the past, although there is variation in the details of available information and the confidence of families in donation history.

Donor records were found for 24 vCJD cases, comprising 20 reported by relatives as blood donors and four additional cases with no reported donation history. Of these, 18 vCJD cases (12% of the total eligible to donate blood) were confirmed to have donated labile blood components, with the number of components made and issued for use in UK hospitals ranging from 1 to 14 per donor. Six vCJD cases were registered as donors, but had not donated labile blood components. Two of these had never attended sessions, three were deferred (due to past medical history, low haemoglobin value and illness, respectively) and one case had donated plasma for fractionation only (made from a single donation from which the red cells were discarded).

The search for donor records was negative in 11 of 31 (35%) vCJD cases reported as putative donors (three of whom allegedly donated well before the onset of the BSE epidemic in the 1980s). The information provided in these negative cases was minimal, except in one case where relatives were confident that regular donations (up to 50) had been made in the years leading up to 1993. Despite extensive searches no records were found; moreover, blood collection sessions had never been made at the purported venue. No explanation has been found for the lack of records, although discrepancies in some of the details given suggest that the history was not as certain as initially thought.

Labile components issued to hospitals

Sixty-six labile components originating from 18 donors were issued to UK hospitals over the period 1981–2004 and transfused to patients according to blood transfusion laboratory records. A further nine components issued between 1982 and 1996 could not be traced by the relevant hospital. Table 1 gives the number of recipients transfused by year and the type of blood component transfused. Fifty-six recipients (85%) received red cells or whole blood, seven (11%) were transfused with labile plasma components or derivatives and three (4%) received pooled platelets made according to UK specifications in which the buffy-coat preparation containing platelets from the implicated vCJD donor was pooled with buffy coats from three other donors and resuspended in plasma from one of the four donations. Nearly half of the red cell recipients received red cells that had been leucocyte-depleted by pre-storage filtration to $< 5 \times 10^6$ leucocytes per unit (in 99% of units with 95% statistical confidence according to UK guidelines [6]) after the introduction of universal leucocyte depletion of the UK blood supply in 1999.

Recipients of blood components

Patient identifiers are available for 66 recipients who received blood from 18 different donors who went on to develop vCJD. None of the 66 recipients had themselves donated blood between receiving their transfusion and early 2004 when the UKBS implemented a policy of excluding all donors transfused in the UK since 1 January 1980. It is of note that 41 (62%) recipients were aged over 60 years at the time of transfusion and were not eligible to donate. All living recipients ($n = 26$) have been informed of their risk and advised not to donate blood, tissues or organs. Three instances of probable transfusion transmitted vCJD infection have occurred, including two confirmed clinical cases and one pre- or subclinical infection. Of these, two cases have died, and one is still alive (see succeeding discussion). Figures 1 and 2 show the survival period for dead (transfusion to death) and live recipients (transfusion to 1 March 2006) of vCJD components, respectively, according to the interval between transfusion and onset of clinical symptoms in the donor.

Dead recipients

Forty recipients (61%) are known to be dead, with mean age at death 66 ± 19 years. Table 2 gives the time and cause of death as stated on death certificates for the recipients known to have died. Around half ($n = 21$) of the dead recipients died within a year of receiving their transfusion, with only seven surviving for more than 5 years. Two recipients, who died 4 months and 14 months, respectively, after transfusion had

