# Preclinical vCJD after blood transfusion in a *PRNP* codon 129 heterozygous patient

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**We report a case of preclinical variant Creutzfeldt-Jakob disease (vCJD) in a patient who died from a non-neurological disorder 5 years after receiving a blood transfusion from a donor who subsequently developed vCJD. Protease-resistant prion protein ($PrP^{res}$) was detected by western blot, paraffin-embedded tissue blot, and immunohistochemistry in the spleen, but not in the brain. Immunohistochemistry for prion protein was also positive in a cervical lymph node. The patient was a heterozygote at codon 129 of *PRNP*, suggesting that susceptibility to vCJD infection is not confined to the methionine homozygous *PRNP* genotype. These findings have major implications for future estimates and surveillance of vCJD in the UK.**

In 2003, an elderly patient in the UK was diagnosed with variant Creutzfeldt-Jakob disease (vCJD) that seemed to have been transmitted by a transfusion of non-leucodepleted red cells from a patient who developed vCJD after the donation.[1] The same investigation also reported 17 individuals alive in December, 2003, who had received labile blood components from donors who subsequently developed vCJD.[1] We report an autopsy detection of a preclinical case of vCJD infection, which appears to have been transmitted by blood transfusion in one of this cohort.

In 1999, an elderly patient received a unit of non-leucodepleted red blood cells from a donor who developed symptoms of vCJD 18 months after donation. The donor died in 2001 and vCJD was confirmed after autopsy. The recipient died 5 years after receiving the transfusion, with no evidence of a neurological disorder. Medicolegal instruction for autopsy was issued. The immediate cause of death was a ruptured abdominal aortic aneurysm. We are bound by a medicolegal restriction regarding disclosure of the patient's age, sex, and geographical location.

We assessed samples of frozen brain, spinal cord, dorsal root ganglion, lymphoid tissues, and muscle for the presence of protease-resistant prion protein ($PrP^{res}$) by western blot with phosphotungstic acid precipitation and the monoclonal antibody 3F4.[2] Immunohistochemistry and paraffin-embedded tissue blotting was done on protease-treated tissue sections from a wide range of tissues, with a panel of four antibodies raised against different epitopes of prion protein (PrP).[3] Restriction fragment length polymorphism analysis of DNA extracted from frozen brain material identified the patient as being heterozygous (methionine/valine) at codon 129 of the prion protein gene (*PRNP*). Consent for full sequence analysis of *PRNP* had not been obtained. Western blot analysis showed the presence of $PrP^{res}$ in spleen (figure 1). The mobility and glycoform ratio of the signals in spleen were similar to those seen in spleen from patients with clinical vCJD and in vCJD brain diluted in non-CJD spleen (figure 1), and were distinct from those described in a subset of sporadic CJD cases, usually with a relatively lengthy clinical illness.[2] We found that $PrP^{res}$ positivity by this method was a consistent feature of four autopsy specimens of spleen from patients who had vCJD, but was absent from a series of nine spleens from controls without CJD (data not shown).

The brain (1337 g) showed only age-related changes, with no pathological features of vCJD. $PrP^{res}$ was undetectable in the brain and spinal cord by western blotting, paraffin embedded tissue blotting, and immunohistochemistry. Immunoreactivity for PrP was found in a few germinal centres in the spleen, in a pattern consistent with staining of follicular dendritic cells (figure 2, A). The number of positive follicles was far lower than in clinical cases of vCJD, with a less aggregated accumulation of immunoreactivity.[3] Immunoreactivity for PrP was also found in a germinal centre within a cervical lymph node, with similar pattern of positivity to that noted in the spleen (figure 2, B). $PrP^{res}$ was not detectable by western

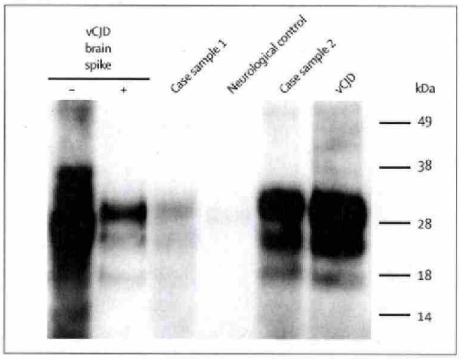


*Figure 1:* **$PrP^{res}$ analysis of spleen by western blot**
Two samples of the patient's spleen were compared with spleen samples from a control with non-CJD neurological disease and from a patient with vCJD, and with vCJD brain homogenate (10 μg) diluted in non-CJD spleen, with (+) or without (–) proteinase K digestion. Every lane represents the phosphotungstate precipitate from 50 mg wet weight of spleen. Horizontal lines indicate positions of molecular weight markers. Amounts of $PrP^{res}$ in eight samples of spleen from the patient were undetectable in two samples (not shown), intermediate in five (sample 1), and similar to those found in the spleen of a patient with vCJD at autopsy in one (sample 2).

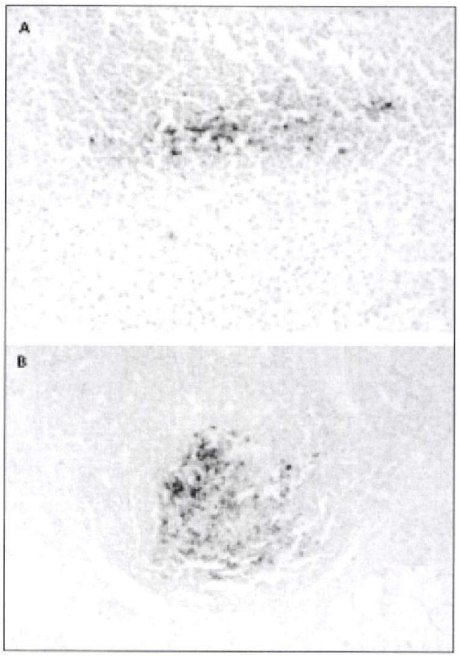


**Figure 2: PrP in germinal centres within the spleen and cervical lymph node**
Germinal centres are labelled (brown) with the anti-PrP antibodies 3F4 in spleen (A) and 12F10 in a cervical lymph node (B) in a pattern similar to that noted in the follicular dendritic cell network, with less aggregated positivity than in cases of clinical vCJD. Original magnifications (A) × 20 and (B) × 10.

blotting in samples of tonsil, another cervical lymph node, dorsal root ganglion, and muscle; neither was it detected in the lymphoid follicles within the tonsil, appendix, and large intestine by immunohistochemistry.

This is the first recorded case in the UK of autopsy detection of preclinical vCJD infection. We have previously shown preclinical PrP immunoreactivity in germinal centres within appendix tissue from two patients who underwent appendectomy 8 months and 2 years before the onset of vCJD.[4] The patterns of PrP accumulation within the germinal centres in the spleen and cervical lymph node in the present case were similar to those seen in three surgically removed appendices from a large anonymised retrospective study, suggesting that these findings might also represent preclinical vCJD infection.[4]

Our findings also show that vCJD infection can be confirmed by western blot analysis of $PrP^{res}$ in an individual who is a heterozygote at codon 129 of *PRNP*.[1,3] This finding has major implications for future estimations of numbers of vCJD cases in the UK, since individuals with this genotype constitute the largest genetic subgroup in the population.[4] This subgroup might have a different incubation period after exposure to either primary infection by the bovine spongiform encephalopathy (BSE) agent or secondary infection by blood transfusion. A very lengthy incubation period might explain why no clinical cases of vCJD have yet been observed in this subgroup. Such preclinical cases might also represent a source of iatrogenic infection themselves, either by blood donation or by contamination of surgical instruments coming into contact with lymphoid tissues, even in the absence of infectivity in the brain.

This patient was a UK resident and might therefore have had dietary exposure to the BSE agent. However, the chance of observing vCJD transmission in the absence of a transfusion infection in a second recipient of blood from a donor with vCJD must be far less likely than the 1 in 15 000 to 1 in 30 000 chance for the first reported case.[1] $PrP^{res}$ was not detected in the nine patients without CJD used as negative controls in this study, and in a previous study we and others did not detect PrP accumulation in lymphoid tissues in 56 cases of other forms of human prion disease and in 85 non-CJD cases.[5] The restriction of $PrP^{res}$ to the spleen and cervical lymph node (but not the tonsil or gut-associated lymphoid tissue) in this case is consistent with an intravenous rather than oral route of exposure. It is also possible that the *PRNP* codon 129 genotype might affect the distribution of PrPres in tissues.

This case highlights the need for continuing surveillance for CJD in the UK, and strongly reinforces the role of the autopsy in the investigation and diagnosis of both clinical and preclinical forms of human prion disease.

#### Conflict of interest statement
None declared.

#### Contributors
A H Peden did biochemical analysis and photography, and contributed to drafting of the manuscript. M W Head did biochemical analysis, and contributed to the drafting of the manuscript. D L Ritchie did histological analysis and photography, and contributed to the drafting of the manuscript. J E Bell did the autopsy, provided the autopsy data, and contributed to the drafting of the manuscript. J W Ironside did the histological analysis and coordinated the preparation and drafting of the manuscript.

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# Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood

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**In 1999, the UK implemented universal leucoreduction as a precaution against transmission of variant Creutzfeldt-Jakob disease by transfusion of domestic blood or red blood cells. We aimed to assess how effectively leucoreduction reduced infectivity of transmissible spongiform encephalopathies (TSEs) in blood. 450 mL of whole blood collected and pooled from scrapie-infected hamsters was leucoreduced with a commercial filter. Blood cell concentrations were quantified, and infectivity titres measured. Blood cell recovery and white blood cell removal complied with American Association of Blood Banks standards. Leucofiltration removed 42% (SD 12) of the total TSE infectivity in endogenously infected blood. Leucoreduction is necessary for the removal of white-cell-associated TSE infectivity from blood; however, it is not, by itself, sufficient to remove all blood-borne TSE infectivity.**

Transmissible spongiform encephalopathies (TSEs) are fatal CNS infections that can incubate asymptomatically for a decade or more in human beings before the appearance of clinical disease. People in the asymptomatic phase of variant Creutzfeldt-Jakob disease (vCJD) appear healthy and donate blood with the same frequency as any healthy person. Transmission of vCJD by transfusion was recently recognised in Great Britain.[1] To reduce the risk of transfusion transmission of such diseases in human beings, the UK implemented universal leucoreduction of donated blood in 1999. This measure was based on the expectation that infectivity would be associated with white blood cells.[2] However, findings in blood from infected mice and hamsters suggested otherwise; at least 40% of the infectivity was plasma-associated, suggesting that leucoreduction would not eliminate infectivity (Rohwer laboratory, unpublished).[3] Other investigations showed no loss of infectivity when small amounts of TSE-infected plasma were passed through scaled-down filters.[4] Similarly, no significant removal of abnormal prion protein was detected when units of human whole blood, spiked with a microsomal fraction from TSE-infected brain, were passed through leucoreduction filters from any of the four major suppliers.[5] Because of reservations about the relevance of these experiments, none of these findings aroused concern.

We investigated the effectiveness of leucoreduction in removal of TSE infectivity from a human-sized unit of pooled hamster blood. To ensure that the 150 hamsters needed for a 450 mL blood pool were at the same symptomatic stage of disease (wobbling gait and head bobbing) for each of two separate experiments, 400 weanling golden Syrian hamsters (Harlan, Madison, WI, USA) were inoculated intracranially with 50 µL of brain homogenate containing about 250 infectious dose$_{50}$ ($ID_{50}$) of hamster-adapted scrapie-strain 263K. A low dose of infectivity was used to preclude re-isolation of the inoculum in the blood. This animal protocol was approved by the University of Maryland Institutional Animal Care and Use Committee.

We obtained two pools of blood from the hamsters, one at 106 days and one at 111 days after inoculation. Under carbon dioxide anaesthesia, 3·5 mL of blood was drawn from the right ventricle into 0·5 mL of CP2D anticoagulant. Care was taken not to touch any other tissue. Only perfect bleeds containing 12·5% CP2D with no visible clots were pooled.

Two in-line leucofiltration systems from Pall Corporation (Port Washington, NY, USA) were evaluated. We selected the Leukotrap WB collection set for the infectivity study because filtration and component separation of hamster blood was fully compliant with American Association of Blood Banks (AABB)[6] specifications, and required only two titrations for interpretation. The Leukotrap RC-PL system

| | Volume (mL)* | White blood cells† | | Red blood cells, total (% of total) | Platelets, total (% of total) |
|---|---|---|---|---|---|
| | | Total (% of total) | $Log_{10}$ reduction | | |
| Whole blood | 448·5 | $2·1\times10^{9}$ (100%) | 0 | $3·7\times10^{12}$ (100%) | $1·4\times10^{11}$ (100%) |
| Leucoreduced blood | 424·2 | $3·0\times10^{6}$ (0·15%) | 2·9 | $3·6\times10^{12}$ (100%) | $1·5\times10^{11}$ (100%) |
| Plasma | 179 | $3·0\times10^{5}$ (0·02%) | 3·8 | 0 (0%) | $1·1\times10^{10}$ (8%) |
| Red blood cells + AS3 | 305·9 | $2·0\times10^{6}$ (0·15%) | 3 | $3·1\times10^{12}$ (86%) | $1\times10^{11}$ (71%) |

*Volume measurements were obtained by weight using experimentally determined densities of whole hamster blood, 1·04 g/mL. †Values are average of at least three separate microscopic determinations using a haemocytometer and by flow cytometric measurements with white cells stained with propidium iodide. AS3 is a preservative and stabiliser.

*Table 1:* **Blood component cell numbers and volumes before and after leucoreduction**

| | Volume inoculated (mL) | Total animals inoculated | Total animals infected | Titre in ID/mL (SD) | Fractional distribution of infectivity |
|---|---|---|---|---|---|
| Whole blood | 5·2 | 104 | 50 | 13·1 (1·6) | 1 |
| Leucoreduced blood | 5·4 | 108 | 34 | 7·6 (1·2) | 0·58 |

Titre and SD calculated from the Poisson distribution as described in the text.

*Table 2:* **Concentration of TSE infectivity in whole and leucoreduced blood**

approached, but did not fully achieve all specifications; furthermore, because more than one filter is involved, more titrations would have been required to evaluate the removal of infectivity.

For the infectivity study, 448·5 mL of CP2D-anticoagulated whole hamster blood was pooled into the whole-blood receiving bag of a Leukotrap WB collection set and processed within the 8-h time limit specified by the AABB. Filtration was done at room temperature under gravity with a 60-inch pressure head on the in-line WBF2 filter, and was completed in 30 min. After removal of a 19 mL sample of the leucoreduced whole blood for subsequent testing, the remainder was centrifuged at 4150 rpm (about 5000 g) for 8 min at room temperature in a Sorvall RC-3C centrifuge. The plasma fraction was expressed into a satellite in-line bag. A preservative and stabiliser, AS3, was added to the red blood cells. Samples of the pre-filtration whole blood, post-filtration whole blood, red blood cells, and plasma were removed for analysis of cell composition and for titration in animals.

Cellular composition of the blood was assessed with a HemaVet five-part differential cell counter calibrated for hamster blood cells (Drew Scientific, Oxford, CT, USA). The residual white blood cell concentrations in the leucoreduced samples were measured by manual count and flow cytometry.

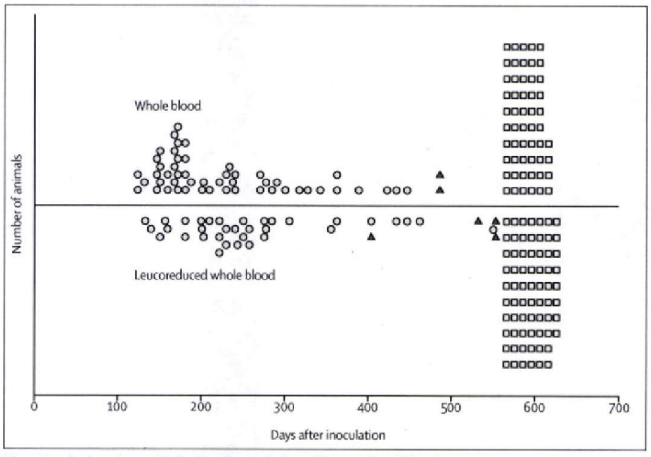


*Figure:* **Incubation times of infections from whole and leucoreduced blood**
Results of inoculations of whole blood are represented by data above the horizontal line; those from inoculations of leucoreduced blood are shown below the line. Circles represent infected animals. Squares represent uninfected animals that survived to the end of the experiment. Triangles represent animals that died intercurrently of causes other than the inoculum.

Infectivity of whole and leucoreduced blood was quantified by limiting dilution titration, a method developed in the Rohwer laboratory. The two samples were processed and inoculated separately and sequentially. Each sample of blood was sonicated with a separate sterile probe to lyse cells and disperse infectivity. It was then immediately inoculated intracranially, 50 µl at a time, into about 100 weanling golden Syrian hamsters that were deeply anaesthetised with pentobarbital. Animals were maintained for 566 days; those that contracted scrapie were killed when the clinical diagnosis was conclusive, and animals still alive at the end of the study were killed. All brains were tested for the presence of the proteinase K-resistant form of prion protein by western blot using 3F4 antibody.

The limiting dilution of an endpoint dilution titration is that at which not all of the inoculated animals become infected. At limiting dilution, the distribution of infectivity into individual inoculations is described by the Poisson distribution, where P(0)=probability of no infections at that dilution and inoculation volume, or (1–probability of infection). From the Poisson distribution $P(0)=e^{-(titre)}$ and titre=–ln(P[0]) expressed as ID/(inoculation volume). SD of the limiting dilution titre is the square root of the titre in ID/mL divided by the total volume inoculated in mL.

Table 1 shows the distribution of cells in each component of the scrapie-infected blood. Leucofiltration reduced the number of white blood cells by 2·9 log, thereby meeting the AABB standard. White cell contamination of the red blood cell fraction and red blood cell recovery were within AABB specifications of less than $5\times10^6$ and greater than 85%, respectively. Hamster platelets are not removed by the WBF2 filter, and partition with the red cells during centrifugation.

The incubation times of infections in each measurement are shown in the figure. At limiting dilution, incubation times begin at the end of the predictable dose response seen in endpoint dilution titrations (about 140 days) and rarely extend beyond 500 days. All clinical and western blot results were consistent.

The limiting dilution titre of the whole blood pool (table 2) was close to the values from titrations of similar pools of whole blood by this method (unpublished data). Leucofiltration of whole blood removed only 42% (SD 12) of the initial TSE infectivity (table 2); of the 5900 ID present in the original unit of blood, 3400 ID were recovered in the leucofiltered blood.

Ideally, leucoreduction would be validated by measuring infectivity concentrations before and after leucoreduction of full units of vCJD-infected human blood. However, it is not currently possible to assay

either infectivity or the infection-specific form of the prion protein in human blood. By contrast, limiting dilution titration of rodent blood can detect less than 1 ID/mL of TSE infectivity and can readily show a difference of less 20% between samples. With this technique we did a study that: avoided the issue of spikes by using endogenously infected blood; avoided the question of scale by using a human-sized unit of fresh hamster blood obtained within the time limits specified for human blood; minimised the possibility of artefact by using a commercial blood collection set with integral filtration unit and a blood centre centrifuge and expressor; and achieved precision in the infectivity measurements by limiting dilution inoculation of 5 mL of each fraction. We assessed the performance of the filter by measuring the level of white blood cell reduction obtained and the cell recoveries of each component. The leucoreduction met or exceeded AABB specifications for all relevant variables.

Leucoreduction removed only 42% of the initial TSE infectivity from whole blood. This distribution is consistent with that obtained in a centrifugal separation of TSE-infected hamster whole blood, in which the buffy coat contained 70% of the total white cells but only 45% of the total whole blood infectivity (unpublished data). Both methods showed that a substantial proportion of the TSE infectivity was not associated with white cells. We have shown previously[7] that TSE infectivity is not associated with highly purified platelets, and we are currently testing purified red blood cells. We presume that the majority of blood-borne infectivity is plasma-associated.

Although leucoreduction is a necessary step for removing white-cell-associated TSE infectivity from blood, this process is insufficient to remove the risk from an infected transfusion unit. Due to the low concentration of TSE infectivity in blood and the absence of screening or inactivation alternatives, removal is an attractive strategy. However, the feasibility of removal depends upon the actual associations and distributions of TSE infectivity in blood itself, which can only be ascertained by assessment of endogenous blood-borne infectivity.

**Contributors**

The overall design and execution of the experiment, including management of the logistics and all the infectivity work, was by L Gregori and R G Rohwer with the assistance of the staff of the Molecular Neurovirology Laboratory. A Giulivi, N McCombie, D Palmer, and P Birch supplied expertise on blood centre operations, blood collection, component separation, leucoreduction, and quantitation of white blood cells. D Palmer and P Birch undertook and interpreted flow cytometry. S Coker supplied expertise on the use of the collection set and leucofilter.

**Conflict of interest statement**

R G Rohwer is a cofounder and part owner of Pathogen Removal and Diagnostics Technologies, which is developing technologies for the removal of TSE infectivity from blood and other materials. L Gregori receives contract support from Pathogen Removal and Diagnostics Technologies for studies on TSE removal. S Coker is an employee of Pall Corporation, which produces leucofilters and is developing TSE removal strategies for blood. The remaining authors declare that they have no competing financial interests.

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