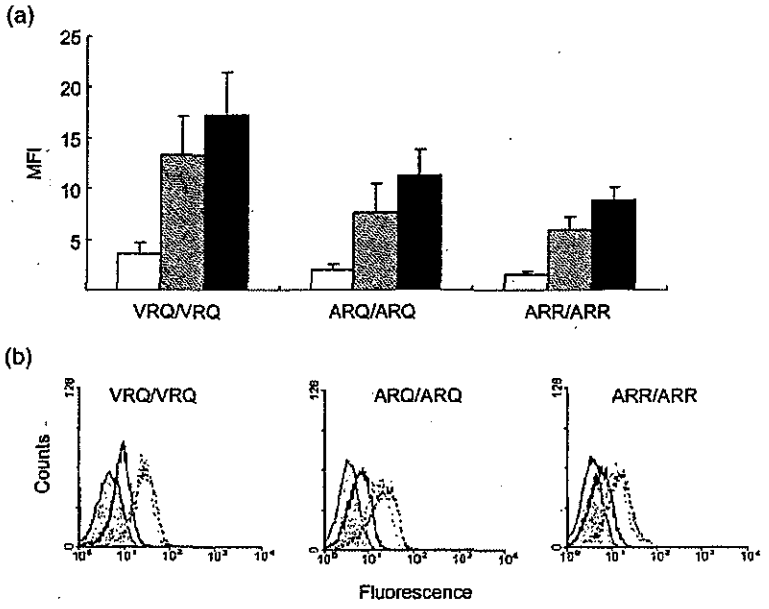

Fig. 2. Effect of PrP genotype on the PrP^C expression level of sheep PBMCs. (a) MFI values (mean ± SD; $n=7$) are shown for PBMCs from uninfected sheep homozygous for VRQ, ARQ and ARR, incubated with FH11 (open bars), 6H4 (hatched bars) and 8G8 (filled bars). (b) Representative flow-cytometry staining profiles for each antibody: FH11 (black line), 6H4 (grey dashed line) and 8G8 (black dashed line). Filled area, isotype-matched negative controls.

Expression of PrP on PBMCs of scrapie-infected sheep

The level of PrP^C expression on PBMC subsets was examined in scrapie-infected sheep with the PrP genotypes VRQ/VRQ (Fig. 4a) and VRQ/ARQ (Fig. 4b) and compared with uninfected sheep of the same genotypes ($n=4$ for each group). In general, scrapie infection appeared to result in higher levels of PrP being detected on the surface of PBMC subsets. Principal-component analysis revealed that the PrP-specific MFI values for $CD62^+$ and $CD21^+$ subsets provided the greatest significance in separating scrapie-infected from uninfected sheep (data not shown). These results suggested that there are increased levels of PrP on PBMCs from some scrapie-infected sheep, particularly on the $CD21^+$ and $CD62^+$ subsets, which warrants further investigation. As antibodies that distinguish PrP^C and PrP^{Sc} (or other disease-associated isoforms) are not readily available, we could not tell whether the increase in cell-surface PrP was a result of accumulation of PrP^{Sc} or altered turnover of PrP^C.

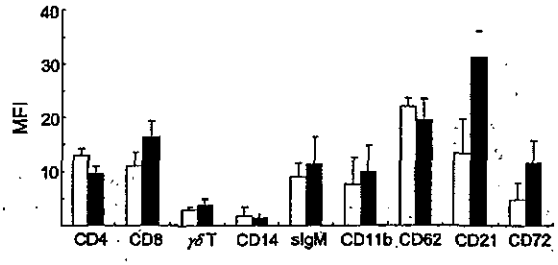

Fig. 3. PrP^C expression on PBMC subsets from uninfected sheep. PBMCs from VRQ/VRQ (open bars) and VRQ/ARQ (filled bars) sheep were analysed by two-colour immunofluorescent staining, using subset-specific mAbs and 8G8. Each bar represents the MFI (mean ± SD; $n=4$) in the FL2 channel (PrP) for each subset.

DISCUSSION

PrP^C is expressed widely in tissues other than the nervous system, although its precise function(s) remains obscure.

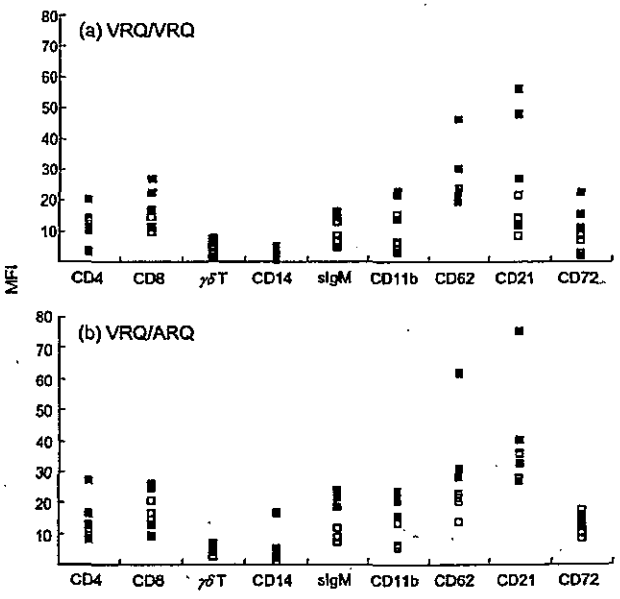

Fig. 4. Comparison of PrP^C expression on PBMC subsets from uninfected and scrapie-infected sheep. (a) VRQ/VRQ sheep; (b) VRQ/ARQ sheep. Individual values for MFI in the FL2 channel (PrP) are shown for uninfected (□) and scrapie-infected (■) sheep ($n=4$ for each group).

The distribution of PrP^C on cellular components of blood shows species-specific variation and our results confirm previous work demonstrating that, in sheep, cell-surface expression of PrP^C is confined to PBMCs. However, in follow-up studies using Western blotting, we have now demonstrated that sheep platelets contain significant amounts of PrP^C in an intracellular location. This is surprising, as sheep platelets have been reported not to express mRNA for PrP (Herrmann *et al.*, 2001). However, it is possible that, in sheep, all PrP^C synthesis takes place in the megakaryocytes from which the platelets are derived. Human platelets have been shown to contain PrP^C in intracellular granules and, after activation, they both up-regulate surface PrP^C expression and release soluble PrP^C (Holada *et al.*, 1998; Perini *et al.*, 1996). The latter is thought to be the source of the large amounts of soluble PrP^C found in human plasma (MacGregor *et al.*, 1999). However, the functional significance of platelet-associated PrP^C remains unclear.

PrP^C expression alone does not appear to be sufficient to render a particular tissue or cell type susceptible to TSE infection, as it is expressed in many tissues that do not become infected during disease (Bendheim *et al.*, 1992). Conversely, although hamster PBMCs do not express PrP^C, infectivity is associated with these cells in hamsters infected experimentally with scrapie (Holada *et al.*, 2002). In this case, it is possible that hamster PBMCs act as carriers of the infectious agent, without being actively infected themselves. Here, we have shown that the level of PrP^C expression on sheep PBMCs correlates with PrP genotype, with the highest levels found on PBMCs from sheep that are highly susceptible to scrapie (PrP genotype VRQ/VRQ) and the lowest levels on scrapie-resistant sheep (PrP genotype ARR/ARR). These results disagree with those published recently by Thackray *et al.* (2004), who concluded that PBMCs from scrapie-susceptible and -resistant sheep expressed similar levels of cell-surface PrP^C. The reason for the discrepancy is not clear, but probably relates to the different mAbs used in their experiments. They measured the binding of two N-terminal mAbs with predicted epitopes between residues 25 and 89 of sheep PrP and one of these mAbs did in fact stain a significantly higher percentage of PBMCs from VRQ-homozygous sheep compared with ARQ and ARR homozygotes. In our experiments, we used three PrP-specific mAbs with epitopes in different regions of the molecule at residues 54–57 (FH11), 95–110 (8G8) and 144–152 (6H4), which did not include the polymorphic residues at positions 136 and 171. The genotype-specific differences in PrP^C staining were observed consistently with each of these antibodies, making it unlikely that the results can be explained by differences in antibody affinity for the variant PrP proteins or subtle differences in protein conformation that might influence the accessibility of certain epitopes to antibody binding. The much lower levels of binding of FH11 compared with the other two mAbs may possibly be explained by expression of an N-terminally truncated form of PrP^C on PBMCs, which would lack the FH11 epitope. Further work will be required to determine whether PrP-dependent differences in PrP^C expression levels extend to other tissues and to elucidate the mechanisms controlling protein levels expressed by different PrP alleles. These could operate at the level of gene transcription and translation, or result from differences in the rate of turnover of different PrP proteins. Thermal-denaturation studies with recombinant ovine PrP proteins have shown that the formation of unfolding intermediates of VRQ and ARQ proteins requires higher activation energy than in the case of ARR proteins, implying that susceptibility-associated PrP variants are intrinsically more stable than the ARR protein (Rezaei *et al.*, 2002). In addition, it has been found that recombinant ARR protein is degraded more rapidly by proteasomes than VRQ protein (Tenzer *et al.*, 2004). Factors such as these might contribute to a more rapid turnover of ARR PrP, leading to reduced cell-surface levels. It is possible to speculate that this could contribute to disease resistance by reducing the opportunity for conversion of ARR protein to the disease-associated isoform.

PrP^C was found on the cell surface of all subsets of PBMCs and there was considerable variation in expression levels between the different subsets. In both VRQ/VRQ and VRQ/ARQ genotypes, PrP^C expression was increased on $CD21^+$ B cells in comparison with cells expressing CD72, a pan-B-cell marker. In peripheral blood of adult sheep, there are two distinct subsets of B cells, which can be distinguished by their expression of the markers CD21, CD62 (L-selectin) and CD11b (Gupta *et al.*, 1998; Young *et al.*, 1997). The $CD21^+CD62^+CD11b^{lo}$ subset recirculates through secondary lymphoid tissues, whereas the $CD21^-CD62^-CD11b^{hi}$ subset is confined to blood and the marginal zone of the spleen. These subsets have been compared with the B1 and B2 cell subsets found in mice, but they do not share all their characteristics and it is unclear whether they represent distinct cell lineages or different maturation stages of B cells. Our results showing high levels of PrP^C expression on $CD21^+$ cells and lower levels on surface IgM^+, $CD72^+$ and $CD11b^+$ cells suggest that the recirculating B-cell subset expresses much more PrP^C at the cell surface than the non-recirculating subset. In addition, high levels of PrP^C expression were found on $CD62^+$ cells, which include α/β and γ/δ T cells as well as $CD21^+$ B cells and also recirculate preferentially through lymph nodes and Peyer's patches.

Further work is required to determine whether PBMC subsets expressing higher levels of PrP^C are more susceptible to scrapie infection and the implications for the peripheral pathogenesis of scrapie. However, a preliminary comparison of PrP expression levels in uninfected and scrapie-infected sheep suggested that, in some infected sheep, very high levels of PrP were found on $CD21^+$ B cells and $CD62^+$ lymphocytes. As we did not have access to mAbs that can distinguish PrP^{Sc} from PrP^C, it is not possible to say whether the apparent increase in protein levels is a result of

PrP^{Sc} accumulation or upregulation of PrP^{C} expression in response to infection. Further investigations using larger numbers of scrapie-infected sheep will be necessary to establish whether this observation is consistent enough to demonstrate statistical significance and whether it reflects the distribution of infectivity.

Following the reports of transmission of TSEs by blood transfusion in sheep (Houston *et al.*, 2000; Hunter *et al.*, 2002) and recent reports of two transfusion-associated cases of vCJD (Llewelyn *et al.*, 2004; Peden *et al.*, 2004), there is considerable interest in using sheep as a model for transmission of vCJD by blood products in humans. To characterize sheep fully as a model species, it is important to establish PrP^{C} expression patterns in the blood of uninfected sheep, as there are marked differences between species in the distribution of PrP^{C} in blood components. The results presented in this paper provide a more detailed picture of PrP^{C} expression on cellular components of sheep blood. In particular, platelets have been shown to contain intracellular PrP^{C}, although it cannot be detected at the cell surface, and in PBMCs, the highest levels of PrP^{C} expression were found on subsets of cells that recirculate preferentially through lymph nodes and Peyer's patches. In addition, expression of PrP on these particular subsets was upregulated dramatically in some scrapie-infected sheep. These findings provide valuable baseline data for studies on the distribution of infectivity and PrP^{Sc} in the blood of TSE-infected sheep and suggest that B-cell subsets may play a significant role in the peripheral pathogenesis of scrapie in sheep.

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研究報告の概要

背景：非常に高感度な dissociation-enhanced lanthanide fluoroimmunoassay （DELFIA）及びフローサイトメトリーが開発され、健常成人供血者の全血及び分離成分における可溶性細胞プリオンタンパク質（PrP^C）発現の特性を明らかにするために用いられている。これまでに、これらの手法を用いて変異型クロイツフェルト・ヤコブ病（vCJD）患者の血液中の PrP 発現及び濃度を評価した研究はない。

試験デザイン及び方法：vCJD 患者、孤発性クロイツフェルト・ヤコブ病（sCJD）患者、非 CJD 神経疾患対照、健常成人の血液について DELFIA を用いて PrP^C を、フローサイトメトリーを用いて細胞由来 PrP を測定した。

結果：DELFIA 解析により、健常成人と比較して全血中の PrP^C 濃度の有意な低下が vCJD 患者（p=0.012）と非 CJD 神経疾患患者（p=0.0004）で認められた。健常成人（p=0.022）及び神経疾患対照（p=0.050）と比較して sCJD 患者で血漿中 PrP^C における有意な上昇が認められた。フローサイトメトリーでは、血小板やリンパ球における PrP の発現や細胞内 PrP のプロテイナーゼ K に対する感受性に有意な差異は認められなかった。健常成人と比較して赤血球中の PrP は、神経疾患対照で有意に低値であった。

結論：CJD 患者と対照群の血液中に認められる細胞遊離型及び細胞由来 PrP には差があり、診断の一助として疾患を分析する上でその他の検査に有用となる可能性がある。

使用上の注意記載状況・その他参考事項等

赤血球M・A・P「日赤」
照射赤血球 M・A・P「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD 等の伝播のリスク

報告企業の意見	今後の対応
Dissociation-enhanced lanthanide fluoroimmunoassay（DELFIA）及びフローサイトメトリーを用いて変異型クロイツフェルト・ヤコブ病（vCJD）患者の血液中の PrP 発現及び濃度を評価したとの報告である。	今後も引き続き、プリオン病に関する新たな知見及び情報の収集に努める。

Variation in concentration of prion protein in the peripheral blood of patients with variant and sporadic Creutzfeldt-Jakob disease detected by dissociation enhanced lanthanide fluoroimmunoassay and flow cytometry

Tim Fagge, G. Robin Barclay, Ian MacGregor, Mark Head, James Ironside, and Marc Turner

BACKGROUND: A highly sensitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) and flow cytometry techniques have previously been developed and employed to characterize soluble cellular prion protein (PrP^c) expression in whole blood and separated components from healthy adult blood donors. No previous studies with these techniques have evaluated the concentration and expression of PrP in the blood of patients with variant Creutzfeldt-Jakob disease (vCJD).
STUDY DESIGN AND METHODS: For blood from vCJD patients, sporadic CJD (sCJD) patients, non-CJD neurological controls, and healthy adults, PrP^c was measured by DELFIA and cell-associated PrP was measured by flow cytometry.
RESULTS: DELFIA analysis identified a significant reduction in the concentration of PrP^c in the whole blood of vCJD ($p = 0.012$) and non-CJD neurological patients ($p = 0.0004$) compared with healthy adults. A significant elevation was found in plasma PrP^c in sCJD patients compared with healthy adult ($p = 0.022$) and neurological controls ($p = 0.050$). Flow cytometry found no significant differences between groups in expression of PrP on platelets and lymphocytes, nor in sensitivity of cellular PrP to proteinase K. Neurological controls show significantly less PrP on red cells than healthy adults.
CONCLUSION: There are differences in free and cell-associated PrP found in blood of CJD patients and control groups, some of which might be useful with other tests in disease profiling as an aid to diagnoses.

The human prion diseases or transmissible spongiform encephalopathies (TSE) are a group of fatal neurodegenerative disorders believed to be caused by a posttranscriptional conformational change in cellular prion protein (PrP) from its soluble form (PrP^c) to pathogenic protease resistant isoform PrP^{Sc}.[1] The most common of these is sporadic Creutzfeldt-Jakob disease (sCJD) but recently a variant form of CJD (vCJD) was identified in the United Kingdom[2] and has been linked to human infection by the bovine spongiform encephalopathy (BSE) agent. The presence of PrP^{Sr} in the peripheral tissues of patients with vCJD[3,4] and recent experimental transmissions of BSE and natural scrapie between sheep by blood transfusion raise the possibility of the potential for iatrogenic transmission in humans by

ABBREVIATIONS: BSE = bovine spongiform encephalopathy; DELFIA = dissociation-enhanced lanthanide fluoroimmunoassay; pK = proteinase K; PrP = prion protein; PrP^c = soluble cellular prion protein; PrP^{Sc} = pathogenic protease resistant prion protein; sCJD = sporadic Creutzfeldt-Jakob disease; TSE(s) = transmissible spongiform encephalopathy(-ies); vCJD = variant Creutzfeldt-Jakob disease.

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blood transfusion.[5,6] This risk has been highlighted by two recent reports of possible transmission of vCJD by blood transfusion,[7,8] which supports the likely presence of infectivity in the blood of preclinical vCJD patients. There is therefore a pressing need for preclinical screening tests, which either identify PrP^{Sc} or are able to identify infected individuals via the detection of reliable surrogate markers to enhance the safety of the blood supply.

The association of infectivity with blood is poorly understood, although there is growing evidence from studies in mice and hamsters that the initial TSE infection is carried by white blood cells (WBCs)[9,10] to lymphoreticular tissues before neuroinvasion of the central nervous system via the sympathetic nervous system.[11-16] Recent reports of the transmission of natural scrapie and experimental BSE between sheep by whole-blood and buffy-coat transfusion support the theory that infectivity is associated with, but not restricted to, the WBC component.[17] Given that levels of detectable PrP^{Sc} and infectivity in peripheral lymphoreticular tissues such as spleen and tonsil in patients with vCJD are 2 to 3 logs lower than levels detected in the central nervous system,[1,18] it is likely that PrP^{Sc} is present at extremely low concentrations in peripheral blood. Attempts to detect PrP^{Sc} in human buffy coat by Western blot have thus far proven unsuccessful.[4] Intracerebral inoculation of human buffy coat into susceptible mouse models has failed to demonstrate infectivity,[19] although this may be a reflection of small numbers of animals used as well as insufficient assay sensitivity.

Tests designed for the detection of PrP^{Sc} in blood would require a high level of sensitivity, probably several logs greater than those already in place for postmortem TSE disease confirmation in humans and slaughterhouse cattle testing. Brown and colleagues,[20,21] basing calculations on studies in rodent TSE models, hypothesized that at most PrP^{Sc} would be present in blood at a level of 100 infectious units per mL of buffy coat, equivalent to 1 to 10 pg per mL; additional studies predict 10 infectious units per mL of peripheral blood,[22] assuming that the ratio of infectivity to PrP^{Sc} found in rodent TSE models is similar to that in vCJD infected humans. Considering these difficulties in detection sensitivity, alternatives to PrP^{Sc} as surrogate markers for TSEs have been explored in the hope that they will provide a test able to distinguish healthy donors from those harboring preclinical TSE infectivity. *Dissociation-enhanced lanthanide fluoroimmunoassay* (DELFIA) technology was employed here to study potential variation in the expression of the normal prion protein isoform (PrP^{c}) in vCJD and sCJD in humans. This technology has been previously employed by us in the detection of prion protein in different blood components of healthy blood donors[23] when we found that two-thirds of all PrP^{c} was associated with the plasma component, one-fourth in platelets (PLTs), and the remainder associated with mononuclear WBCs and red cells (RBCs). We also showed that PrP^{c} is released from PLTs into plasma.[24] A similar approach has also been used in a report of an increase in PrP^{c} expression in plasma of patients with sCJD and neurological controls when compared with healthy blood donor controls.[25] The expression of PrP in the peripheral blood of patients with vCJD has not been previously studied. In this study, we report levels of PrP^{c} in whole blood and separated blood components of vCJD patients, sCJD patients, healthy adult controls, and neurological controls as measured with DELFIA. In addition, flow cytometric analysis of cell surface PrP expression on different blood cell types was carried out on fresh whole-blood samples from the same clinical and control groups.

MATERIALS AND METHODS

Collection of blood samples

Anonymized whole-blood samples from 30 healthy adult apheresis PLT donors were collected by the Scottish National Blood Transfusion Service Edinburgh and stored for 24 hours at 4°C before separation to mimic the conditions of collection of samples from CJD patients transported to the National CJD Surveillance Unit from around the country. Blood from CJD patients and neurological controls was left over from samples obtained by the National CJD Surveillance Unit for genetic analysis. Samples were not obtained solely for this study owing to the difficulties of obtaining blood from these patients. Whole-blood samples from 10 vCJD patients, 10 sCJD patients, and 8 neurological controls were used for DELFIA studies. Informed consent was obtained from patients and donors for experimentation and ethics approval for the study was obtained from the Scottish Multi-Center Research Ethics Committee. All vCJD and sCJD cases had a probable or definitive diagnosis based on internationally established criteria.[26,27] Neurological controls were samples referred to the CJD Surveillance Unit from patients who subsequently did not meet criteria for a diagnosis of definite or probable CJD. These 8 patients were subsequently diagnosed with neurological disorders distinct from CJD including Alzheimer's disease (2), paraneoplastic syndrome (2), mitochondrial disease (1), Lewy body dementia (1), nonorganic depression (1), and central pontine myelinolysis (1).

All whole-blood samples were handled, separated, and stored in the same manner to ensure groups were directly comparable in scientific investigations. Whole-blood samples were collected into 9-mL vacuettes containing 1 mL of 3.2 percent trisodium citrate (Greiner Bio-One Ltd, Gloucestershire, UK). Blood for flow cytometry (0.4 mL) was set aside fresh as samples arrived at the National CJD Surveillance Unit and tested immediately. Samples were then separated into whole-blood, PLT-poor plasma, RBC, PLT, and buffy-coat components for

archiving frozen for these and other studies. The separation protocol was designed to produce these components without the need to use Ficoll. Ficoll, like dextran sulfate, is a polyanion, and it was thought that it may interfere with the processing and replication of the infectious agent as has been reported for dextran sulfate.[28] A quantity of 1.5 mL of whole blood was transferred to a sterile 2-mL tube, and the remaining volume was centrifuged at 450 × *g* for 10 minutes in a centrifuge (Model 4-15C, Sigma Aldrich, UK).

The PLT-rich plasma was removed and centrifuged at 16,060 × *g* for 10 minutes in a centrifuge (Heraeus Biofuge, Kendro Laboratory Products, Sollentum, Germany); the resulting PLT-poor plasma supernatant was transferred to 2-mL storage tubes. The buffy coat and the RBCs were transferred into separate 15-mL tubes, and 10 mL of phosphate-buffered saline (PBS; Sigma Aldrich, P4417) was added before the tubes were spun at 180 × *g* for 10 minutes. The supernatant and the top 0.5 mL of the interface from the RBC tube were discarded, PBS was added to the 14-mL mark, the tube was centrifuged at 180 × *g* for 10 minutes, and the supernatant was discarded. The pellet was resuspended in PBS to double the volume giving a 50 percent solution of cells, which were transferred to 2-mL storage tubes. The supernatant from the buffy-coat tube was centrifuged at 16,060 × *g* for 5 minutes to pellet the PLTs. The supernatants were discarded and the pellets were washed with PBS, resuspended in 1 mL of PBS, and transferred to a 2-mL storage tube. The buffy-coat pellet was washed in 14 mL of PBS, the supernatant was discarded, and 13 mL of distilled water added to shock-lyse the RBCs. One milliliter of 10× PBS was added before centrifugation at 180 × *g* for 10 minutes. The supernatant was discarded and the pellet was washed with PBS and centrifuged at 100 × *g* for 10 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL of PBS. All separated components were stored at −80°C. Protease inhibitors were not used in the preparation of these components.

DELFIA

The highly sensitive method of time-resolved DELFIA was employed for the detection of PrP^c by sandwich immunoassay with methods described previously by our group.[29] The assay was calibrated with a dilution series of an expired PLT concentrate as a source of human PrP^c. This had been calibrated as a standard with recombinant human soluble PrP (Prionics, Zurich, Switzerland); 1 unit was found to be equivalent to 26 pg per mL. In addition a quality control plasma sample was included to confirm assay reproducibility. Blood component aliquots were thawed and assayed at five dilutions in duplicate. Suitable dilutions for each component were established in earlier experiments (data not shown) to ensure parallelism between dilutions. The means, median, and standard deviation were calculated and the U test was used to determine the significance of differences between groups.

Total protein measurement

Data were expressed in units per milligram of total protein for whole blood and all separated components to normalize data by compensating for the effects of the separation protocol upon cellular particulate recovery in components and to ensure that range differences in cell number did not contribute to differences in PrP^c levels between groups. Although studies on full blood counts of clinical patient samples and controls did not show abnormalities, full blood count data could not be generated from frozen stored clinical and control samples used in this study; therefore, normalization by total protein was essential for all components with the exception of whole blood.

Measurements of total protein levels were carried out with a protein assay (Bio-Rad, Hemel Hempstead, UK). The microtiter plate format was performed following the manufacturers instructions.

Flow cytometry

Blood samples. Samples were processed by flow cytometry immediately on arrival at the National CJD Surveillance Unit and subsequently assigned to clinical groups when diagnoses were made. An aliquot (0.4 mL) of whole blood from each sample was made available for flow cytometry studies. Any samples that did not fall into the diagnostic categories were not included in the analysis of results. Blood samples were also obtained from healthy adults described above and, because clinical samples were at least 24 hours old before arriving in the laboratory, these healthy adult control samples were processed by flow cytometry on the day following sampling.

Treatment with proteinase K. A quantity of 0.2 mL of the sample was set aside for staining (untreated) and the remaining 0.2 mL was washed once in 2.5 mL of cold cell wash (Becton Dickinson, Franklin Lakes, NJ), centrifuged down, and resuspended in 0.5 mL of proteinase K (pK) (Sigma Aldrich) at 1 mg per mL in Hanks' balanced salt solution with calcium and magnesium (Sigma Aldrich) and left at room temperature for 30 minutes (the pK concentration for complete removal of cellular PrP from healthy human blood cells under these conditions was determined by preliminary titration). The pK-treated sample was washed four times in 2.5 mL of cold cell wash, and the resultant cell pellet was divided equally between two 12 × 75-mm Falcon tubes (Becton Dickinson) for flow cytometry staining.

Flow cytometry staining and analysis. This was carried out essentially as previously described[29,30] except that a combination of monoclonal antibodies (MoAbs)