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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^{Sc} 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,[4,18] 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.[23] 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)

3F4 and 4F2 was used on the hypothesis that although MoAb 4F2 clearly identifies PrP on human RBCs whereas MoAb 3F4 does not bind to human RBCs,[28] the 4F2 epitope on any PrP^{Sc} may be more susceptible to pK cleavage than the 3F4 epitope. These MoAbs (5 µL each at 0.5 mg/mL) were added simultaneously to 100 µL of whole blood or pK-treated washed blood with 100 µL of cell wash. Second samples of whole blood and pK-treated blood received 100 µL of cell wash without anti-prion MoAbs as unstained (negative) control. After incubation and washing, anti-mouse immunoglobulin (human absorbed fluorescein isothiocyanate [FITC]-conjugated goat anti-mouse immunoglobulin $F(ab')_2$, Biosource, Camarillo, CA) at 1 in 500 in cell wash (100 µL) was added to all tubes. After incubation and washing, a sample (10 µL) was removed from each tube for RBC studies, and 5 µL of peridinin chlorophyll protein–conjugated anti-CD45 MoAb and 5 µL of phycoerythrin (PE)-conjugated anti-CD41 MoAb (Becton Dickinson) were added to each whole-blood tube. To each RBC study sample, 5 µL of PE-conjugated anti-glycophorin A MoAb (Serotec, Oxford, UK) was added. Samples were incubated an additional 30 minutes in the dark at room temperature. The RBC samples were washed twice with 2.5 mL of cell wash and resuspended in 0.5 mL of cell fix (Becton Dickinson). The whole-blood samples were resuspended in 2.5 mL of lysing solution (Becton Dickinson) to remove RBCs followed by two washes in 2.5 mL of cell wash and resuspended in 0.6 mL of cell fix. Fixed stained samples were kept overnight at 4°C in the dark before analyzing by three-color flow cytometry as previously described.[30] Samples from lysed preparations were collected on linear forward- and side-scatter axes for WBC studies, and a second set was collected on logarithmic forward- and side-scatter axes for PLT studies. RBCs were collected on linear forward- and side-scatter axes. A total of 20,000 events in appropriate forward- and side-scatter gates were collected to listmode files for each analysis. Analysis was conducted with computer software (FCS Express, DeNovoSoftware, Thornhill, Ontario, Canada) on a personal computer. Samples were gated by appropriate forward- and side-scatter patterns and additionally by CD45 expression (different WBC populations), CD41 expression (PLTs), or glycophorin A expression (RBCs). Other markers of WBC populations (e.g., CD14) were susceptible to pK digestion and were not used. Histograms were made of channel-1 (FITC) expression for negative controls (no anti-PrP MoAbs) and overlaid with histograms for channel-1 for anti-PrP (primary anti-PrP MoAbs) for each cell class: single peaks were observed in each case and their median fluorescence intensities determined by setting appropriate markers on the histograms. Net median fluorescence intensity for anti-PrP staining was obtained by subtracting the negative control (no anti-PrP MoAbs) value.[30]

Statistical analysis

Clinical groups were compared with the U test carried out with computer software (NCSS 2001 software, NCSS, Kaysville, UT). Box plots were produced by the NCSS 2001 software according to a common procedure in which boxes represent the interquartile range (IQR), the top and bottom of the box are the 25th and 75th percentiles, and the horizontal line through the box represents the median. The line and bar (whiskers) above and below the box represent the upper and lower adjacent values. The upper adjacent value is the largest observation that is not greater than the 75th percentile plus 1.5 times the IQR. The lower adjacent value is the smallest observation that is at least the 25th percentile minus 1.5 times the IQR. Outliers, shown as small circles, are those values that lie outside the upper and lower adjacent values.[31]

RESULTS

Detection of whole-blood PrP^c by DELFIA

Whole-blood samples from 10 patients with vCJD, 10 patients with sCJD, 8 neurological controls, and 29 healthy adults (1 excluded owing to lack of parallelism between dilutions) were analyzed in duplicate at five dilutions by DELFIA. For each group the median and IQR were calculated (Fig. 1A; Table 1). There is a significant decrease in the concentration of PrP^c in vCJD ($p = 0.012$) and neurological control patients ($p = 0.0004$) compared with healthy adults, but not between vCJD and sCJD, nor between neurological controls and sCJD patients. Despite the significance, there was considerable overlap between the vCJD and healthy adult control groups, which indicates that this observation would have little use as a discriminatory test for diagnosis or screening. These differences between groups were retained and continued to be significant when PrP^c concentration is not normalized for total protein but is expressed directly as units per milliliter; there remains a significant decrease in the concentration of PrP^c in vCJD ($p = 0.005$) and neurological control patients ($p = 0.0001$) when compared with healthy adults.

Detection of PLT-poor plasma PrP^c by DELFIA

PLT-poor plasma samples from 10 patients with vCJD, 10 patients with sCJD, 29 healthy adults (1 excluded owing to lack of parallelism between dilutions), and 6 samples (2 samples unavailable) from neurological controls were analyzed by DELFIA for PrP^c (Fig. 1B; Table 1). We found significant elevation in the plasma PrP^c concentration in sCJD patients when compared with both healthy adult ($p = 0.022$) and neurological control groups ($p = 0.050$), but not when compared to levels found in vCJD patients. No significant differences were found in comparisons

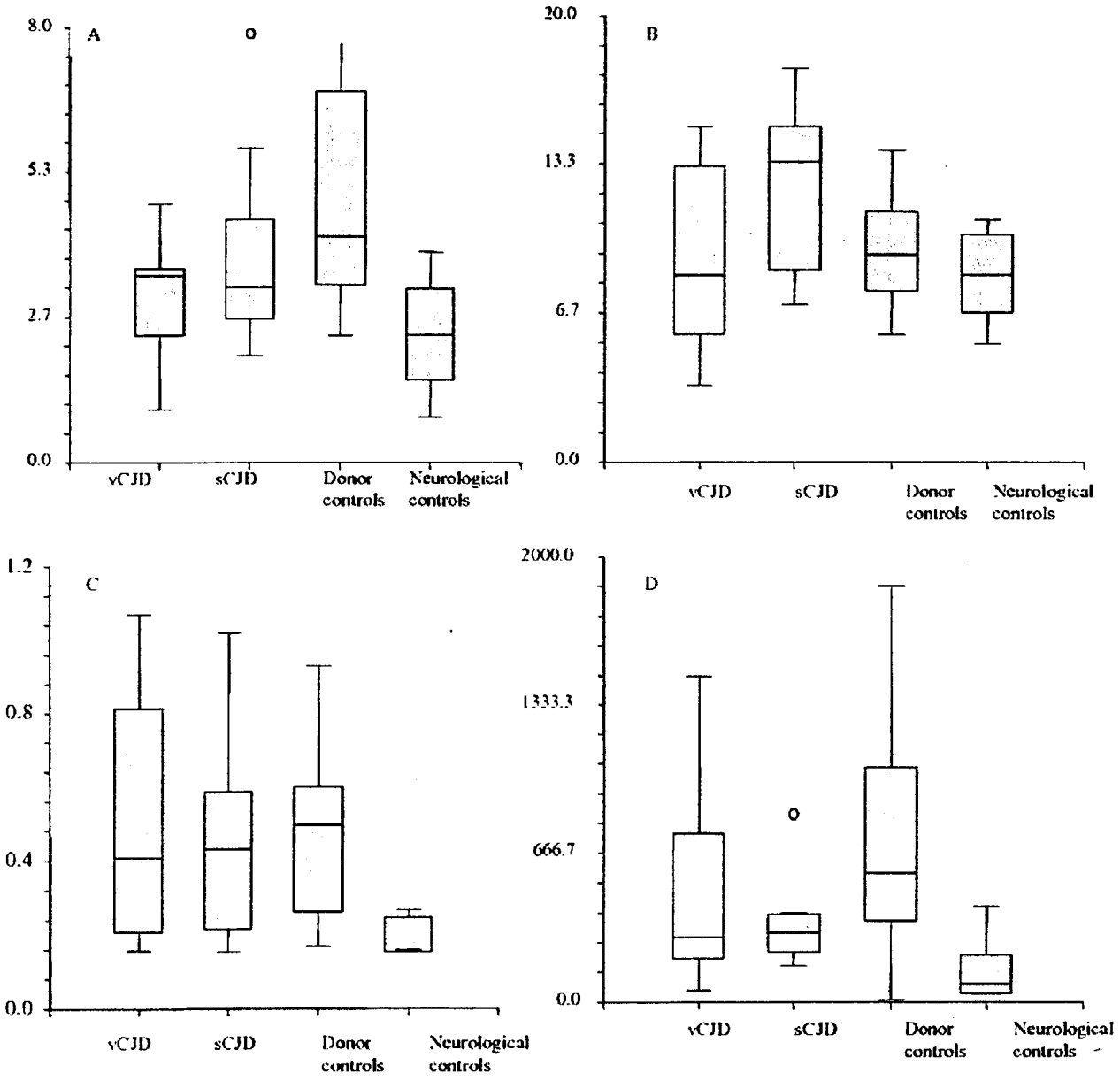

Fig. 1. The concentration of PrP^c assayed by DELFIA in separated blood components of clinical and control samples. The concentration of whole blood (A), PLT-poor plasma (B), 50 percent RBCs (C), and PLT (D) PrP^c (units per mg) assayed by DELFIA in vCJD, sCJD, and donor and neurological control groups.

between controls and vCJD groups. Again, there is considerable overlap of ranges for all groups. Significant differences between groups were not evident when data was expressed in units per milliliter of PrP^c.

Detection of RBC PrP^c by DELFIA

RBC samples from 10 vCJD patients, 9 sCJD patients, 26 healthy adults, and 7 neurological controls were analyzed by DELFIA to determine PrP^c concentration (Fig. 1C; Table 1). vCJD and sCJD patients and healthy adults all exhibited similar median values for RBC PrP^c concentration and therefore did not exhibit significant differences when compared with each other. The neurological control group showed a significant reduction in RBC PrP^c concentration when compared with vCJD patients ($p = 0.029$), sCJD patients ($p = 0.024$), and healthy adults ($p = 0.001$).

TABLE 1. Medians and range (95% confidence limits [CL]; U/mg) of separated components from different clinical and control groups as measured by DELFIA

Sample	Blood component	Number	Median (U/mg)	95% CL (U/mg)
vCJD	Whole blood	10	3.438	1.912-3.593
sCJD	Whole blood	10	3.227	2.073-5.754
Donor controls	Whole blood	29	4.16	3.376-6.635
Neurological controls	Whole blood	8	2.35	0.823-3.375
vCJD	Plasma	10	8.375	5.225-14.912
sCJD	Plasma	10	13.446	8.078-17.314
Donor controls	Plasma	29	9.316	8.038-10.624
Neurological controls	Plasma	6	8.405	5.31-10.8
vCJD	RBCs	10	0.411	0.19-1.045
sCJD	RBCs	9	0.434	0.162-0.648
Donor controls	RBCs	26	0.499	0.29-0.579
Neurological controls	RBCs	7	0.16	0.16-0.27
vCJD	PLTs	9	293.93	174.42-1150.442
sCJD	PLTs	9	316.56	213.69-400.75
Donor controls	PLTs	24	581.11	389.29-855.15
Neurological controls	PLTs	7	83.37	35.48-429.6

Detection of PLT PrP^c by DELFIA

PLT samples from 24 healthy adults, 9 patients with vCJD, 9 patients with sCJD, and 7 neurological controls were analyzed for PrP^c concentration (Fig. 1D; Table 1). Samples excluded from each group constitute those where PrP^c concentration could not be measured reproducibly across a range of dilutions. The concentration of PLT PrP^c in the sCJD samples was significantly reduced compared with levels in healthy adults ($p = 0.021$) but not against vCJD. The level of PLT PrP^c in neurological controls was the lowest of all groups, and this reduction was significant compared to levels in healthy adults ($p = 0.001$), vCJD patients ($p = 0.039$), and sCJD patient samples ($p = 0.017$).

Detection of buffy-coat PrP^c by DELFIA

Buffy-coat PrP^c expression levels were not detected at significantly high concentrations, particularly in clinical groups, to permit accurate measurement. Hence, they were excluded from the analysis.

Flow cytometry

The cell-associated PrP (net median fluorescence intensity above background) ranges for lymphocytes, PLTs, and RBCs are shown as interquartile box plots (Fig. 2) for healthy adult controls, neurological controls, sCJD cases, and vCJD cases. The only significant difference found between groups was for RBC PrP in healthy adults compared to non-CJD neurological controls ($p = 0.008$). Although PLT PrP was increased in both sCJD and vCJD cases compared to healthy adults or neurological controls, this did not reach significance. Clinical vCJD and sCJD cases were found to be as sensitive as healthy adults and non-CJD neurological patients to removal of cell-associated PrP by pK (Fig. 3). The distribution of FITC fluorescence after pK treatment was homogeneous, showing a single low peak in the FITC channel without any discernible higher peak, which might indicate the retention of protease K-resistant PrP, putative PrP^{Sc}, on any subpopulation of cells.

Results are not reported for neutrophils or monocytes because in these studies we considered the results unreliable. There appeared to be considerable nonspecific binding of fluorescence by both WBC classes especially evident in both healthy adult controls and all clinical cases, which may have been a consequence of sample age.

Stability of whole-blood PrP^c

Some samples from clinical groups took longer than 24 hours to arrive in the laboratory. To consider any effects of prolonged transit time at ambient temperature, whole-blood samples from three healthy adult controls were left at 22°C for 72 hours, and samples were removed from each control at 24-hour intervals. Samples were analyzed at each time by flow cytometry, and 1-mL samples for DELFIA analysis were stored at -80° C until analysis for the detection of whole-blood PrP^c. The expression of PrP on PLTs measured by flow cytometry at 24-hour intervals showed sequentially decreasing levels of PrP with time for each individual (not shown). This contrasted with identification of increased expression of PrP on PLTs found in a preliminary analysis of data for sCJD patients compared with healthy adult controls, and implied sample storage was not responsible for this increase in the patient group. Levels of PrP^c detected by DELFIA remained stable across 72 hour (not shown). Prolonged transit time and ambient storage temperatures had negligible effects on PrP^c concentration and are therefore unlikely to contribute to differences between sample groups.

Relationship between age and PrP^c levels

Differences between control and clinical sample groups may be affected by the age of individuals, considering that vCJD usually affects young people and sCJD older people. The concentration of PrP^c (U/mg) in whole-blood samples detected by DELFIA in CJD and control groups was plotted against age and showed that PrP^c expression is unrelated to this variable (data not shown).

Hematology of clinical and control samples

To ensure that hematologic abnormalities in blood samples from clinical patients and controls did not contribute

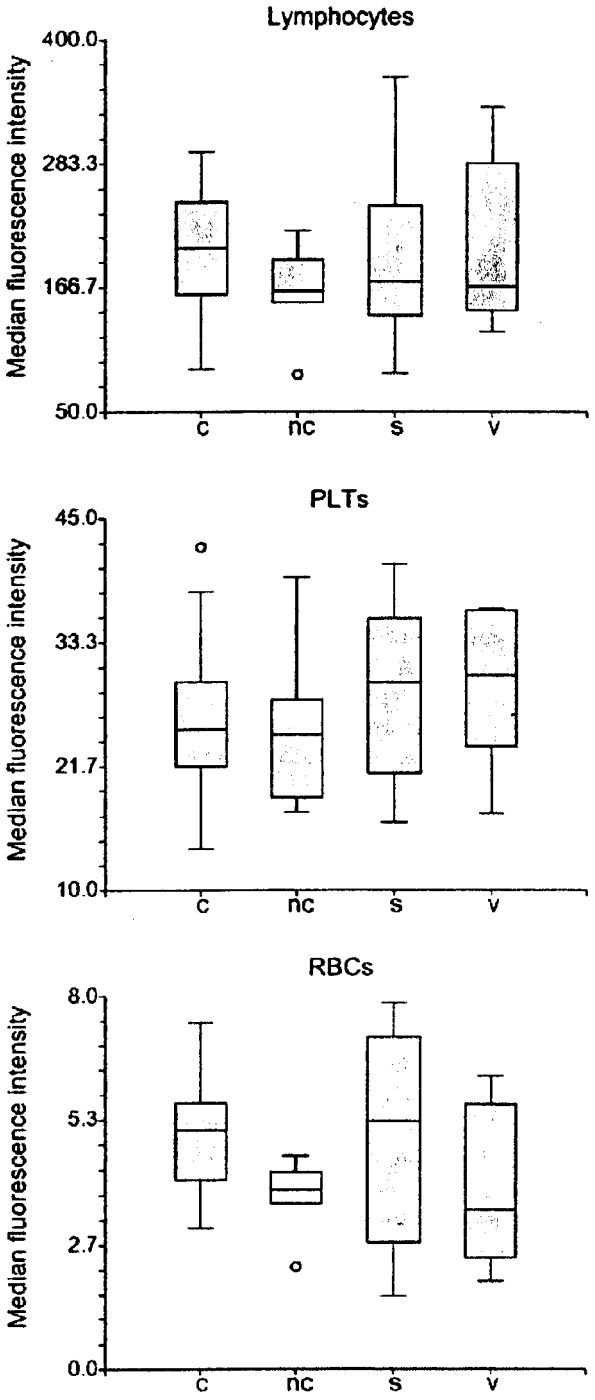

Fig. 2. Box plots showing the ranges of expression of cellular PrP shown by flow cytometry on different clinical and control groups. Expressed as median fluorescence intensity net of background, on lymphocytes, RBCs, and PLTs.

to differences in PrP concentration and expression between groups, full blood count data for clinical and neurological patients were reviewed. No gross abnormalities were detected (data not shown).

DISCUSSION

The DELFIA technique employed here, and described previously by us,[23] is well suited to the screening of whole blood and separated components of clinical and control samples for PrP^c. The assay is quick and has a high sensitivity for the detection of PrP^c. DELFIA technology is compatible with high throughput and has a high signal to background ratio, owing to background deteriorating rapidly whereas the signal has a long lifetime. Flow cytometry analysis has been used previously by us to illustrate the expression of PrP on healthy adult human blood cells.[29,30]

No previous studies have evaluated the concentration and expression of PrP in the whole blood and separated components of patients with vCJD. DELFIA analysis of separated components found that the highest levels of PrP^c associated with the PLT and plasma components and much lower levels associated with RBCs, which is in agreement with our previously described findings.[23] Levels of PrP^c in the WBC buffy-coat fractions were very low, often at the detection limit of the assay, and these levels could not be accurately calculated particularly in clinical groups, hence their exclusion from further analysis. The separation method used here was a compromise for greatest utility of archived samples and was not ideal for isolation of the PLT and buffy-coat components; cells often clumped together and proved difficult to resuspend, which may explain the large median ranges in the PLT data and the low levels of detectable PrP^c in buffy coats. These large ranges in PLT PrP^c concentrations are not supported by any large fluctuations in PLT counts as seen in hematologic data for patients and controls used in this study; the data does not show any abnormalities.

In our analysis of whole blood, we found a significant decrease in the concentration of PrP^c in vCJD and neurological control patient samples compared to healthy adults but not when compared with the sCJD group. We found significant elevation in the plasma PrP^c concentration in sCJD patients when compared with both healthy adult and neurological control groups; however, no significant differences were evident between levels in sCJD and vCJD patients. The elevation of plasma PrP^c in sCJD patients agrees with a previously published report; however, in our study a distinct finding is that levels of plasma PrP^c in sCJD patients are significantly elevated not only against healthy adults but also against the neurological control group, which was not the case in the previous published findings.[25] These differences may be a consequence of our use of neurological controls with conditions, apart from Alzheimer's disease, that were distinct from those

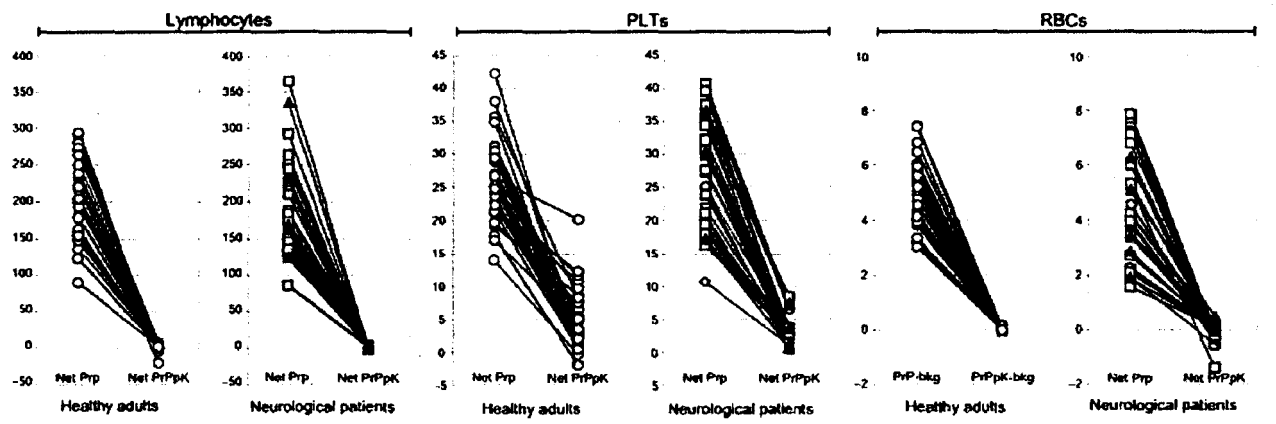

Fig. 3. Demonstration by flow cytometry of the removal of cell-surface PrP after pK treatment. The median fluorescence intensity (net of background) is plotted for cells before and after pK treatment and connected by a line for each case. (○) Healthy adult blood donors; (○) non-CJD neurological patients; (□) sCJD patients; (▲) vCJD patient; (◇) familial TSE (a single case of familial TSE was included in the study but not included in statistical analysis).

included in the previous study. We found a significant reduction in the concentration of RBC PrP^c in neurological controls compared with all other groups. We considered that the differences between groups might be a consequence of the age of patients affected by vCJD, sCJD, and neurological disorders; however, no relationship between age and PrP^c concentration was found in either control or clinical sample groups. It is possible that the reduced concentration of whole-blood PrP^c in vCJD patient samples might be due to the conversion of PrP^c to PrP^{Sc} as part of the replication process involved in disease pathogenesis. The absence of a similar reduction of whole-blood PrP^c in sCJD patients would be expected because PrP^{Sc} is largely confined to the central nervous system and replication of PrP^{Sc} in the periphery before neuroinvasion is not a feature of sCJD. Alternatively, the vCJD whole-blood reduction could be a summation of reductions in PrP^c in the plasma and PLT components, whereas in sCJD the low PLT PrP^c value is offset by the high plasma value so balancing out in whole-blood levels. It is likely that the reduced levels of whole-blood PrP^c in neurological controls is a consequence of the reduced PrP^c levels found in RBCs and PLTs in this group. To some extent the low levels of PLT PrP^c may reflect neurological disease because PrP^c levels were low in PLT samples from CJD and lowest in neurological controls but not in healthy adult controls. We are unable though to speculate on the reasons for this.

We also considered the possibility that increases in sCJD plasma PrP^c levels may be a consequence of its release from PLTs, because PLTs are known to express PrP^c on the cell surface and to store PrP^c in alpha granules, which are released into plasma in a soluble form on PLT activation.[24,32] To support this theory, we have found a significant reduction in the PLT PrP^c concentration in sCJD patient samples compared to healthy adults, implying that PLTs may have shed PrP^c into plasma. The expression of PrP on PLTs of vCJD and sCJD as determined by flow cytometry was elevated compared with healthy adult and neurological controls, and although this elevation did not reach significance, perhaps this represents up regulation of PrP before its release into plasma. Investigations into cell-surface PrP on PLTs did not implicate blood sample storage and transit as a possible cause for increased cell-surface PrP; they found cell-surface PrP on PLTs decreased with sample age.

Flow cytometry analysis demonstrated significantly less PrP expression on RBCs of neurological controls than healthy controls. This finding supports that of a reduced concentration of RBC PrP^c in this group found by DELFIA. These results would appear to indicate that there is less PrP^c per RBC compared with other groups. We are unable to explain the reason behind the finding of this reduced expression of PrP on RBCs. We did not find any evidence of a reduction in the expression of PrP on lymphocytes of patients with sCJD when compared to neurological controls in contrast to a previous report.[33] We used both 4F2 and 3F4 anti-PrP MoAbs together for these flow cytometry studies, however, whereas the previously reported study used 3F4 alone, which does not to bind to human RBCs.[29] It could be possible that MoAb 3F4 might show reduced binding to PrP^c on lymphocytes in some clinical conditions if PrP^c expression were altered or PrP^c interacted with other membrane components because of lymphocyte activation.[34,35] PrP on RBCs, PLTs, and lymphocytes was removed to background levels after pK treatment in all clinical and control groups, indicating the absence of any detectable cellular expression of abnormal disease associated forms of PrP. Our findings indicate that a reduction

in concentration of whole-blood PrP^c may be common in vCJD and other neurological diseases but not sCJD. An elevated level of plasma PrP^c may be common in sCJD. These differences between groups appear genuine, presumably reflecting differences in the disease process in the patients in these particular groups and not an artifact of age, specimen collection, storage, or analysis. Despite the significance of differences between groups, the variations in values are large and there is considerable overlap between CJD groups and control groups, which rules out the exploitation of these differences in whole blood and plasma in screening strategies. These studies expose the limitations in the use of blood PrP^c levels as a diagnostic tool. They illustrate, however, important observations on the distribution of PrP^c in the peripheral blood of CJD patients and the potential of DELFIA-based PrP assays in clinical practice. The analytical sensitivity of DELFIA-based assays used here represents a significant step toward the development of DELFIA for the detection of PrP^{Sc}, which is a much more reliable indicator of infection and is directly associated with infectivity. The current study emphasizes the need to develop assays for its detection in blood.[36,37]

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