Prion proteins in subpopulations of white blood cells from patients with sporadic Creutzfeldt-Jakob disease

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Abstract

Recent cases of prion transmission in humans following transfusions using blood donated by asymptomatic variant Creutzfeldt-Jakob disease (CJD) patients implicate the presence of prion infectivity in peripheral blood. In this study, we examined the levels of the normal, cellular prion protein (PrP\text{C}) and the disease-causing isoform (PrP\text{Sc}) in subpopulations of circulating white blood cells (WBC) from sporadic (s) CJD patients, age-matched neurological controls and healthy donors. Though widely distributed, the highest levels of PrP\text{C} were found in a subpopulation of T lymphocytes: ~12,000 PrP\text{C} molecules were found per CD4\text{+}CD45RA\text{-}CD62L\text{-} effector memory T helper cell. While platelets expressed low levels of PrP\text{C} on their surface, their high abundance in circulation resulted in the majority of PrP\text{C} being platelet associated. Using quantitative FACS analysis, we found that neither WBC composition nor the amount of cell-surface PrP\text{C} molecules was altered in patients dying of sCJD. Eight different WBC fraction types from the peripheral blood of sCJD patients were assessed for PrP\text{Sc}. We were unable to find any evidence for PrP\text{Sc} in purified granulocytes, monocytes, B cells, CD4\text{+} T cells, CD8\text{+} T cells, NK cells, non-classical \gamma\delta T cells, or platelets. If human WBCs harbor prion infectivity in sCJD patients, then the levels are likely to be low.

Keywords

Assay; blood; Creutzfeldt-Jakob disease; detection; prion; WBC

Prions cause a group of fatal, rapidly progressing neurodegenerative diseases, including Creutzfeldt-Jakob disease (CJD) in humans. Sporadic (s) CJD accounts for approximately 80% of all CJD cases worldwide (1). In the late 1990s, an outbreak of a new form of CJD,
termed variant (v) CJD, occurred in Europe, predominantly in England. Since then, nearly 200 cases of vCJD have been identified worldwide and studies indicate that vCJD is caused by consumption of contaminated animal products from cattle with bovine spongiform encephalopathy (BSE) (2).

All prion diseases are caused by the accumulation of an aberrantly folded isoform, denoted PrP<sup>Sc</sup>, of the prion protein (PrP). Encoded by the *PRNP* gene in humans, PrP is a glycosylated membrane protein distributed predominantly on neurons. PrP was first isolated from the brains of hamsters inoculated with an adapted strain of sheep prions (3, 4). Later, genomic sequencing of genetic CJD cases revealed nonconservative mutations in the *PRNP* gene of affected family members (5, 6). With a basic amino acid composition and an unstructured N-terminus, PrP can adopt at least two metastable conformations: (1) native, α-helix-rich PrP<sup>C</sup> and (2) disease-causing, β-sheet-rich PrP<sup>Sc</sup> (7). The latter is self-perpetuating, and continues to recruit and convert more PrP<sup>C</sup> molecules into PrP<sup>Sc</sup> oligomers, which can assemble into amyloid plaques in the brain. Events that catalyze the refolding of PrP<sup>C</sup> into PrP<sup>Sc</sup> have eluded molecular characterization thus far, but the conversion is often accompanied by decreased solubility as well as increased resistance to protease digestion (8).

Although prion infectivity is most readily isolated from the CNS, it can also be found widely distributed among extraneuronal tissues, especially in patients with vCJD (9-11). This distribution in part is due to the ubiquitous expression pattern of PrP<sup>C</sup>, which is required for prion infection. In blood, for instance, PrP<sup>C</sup> is mainly found in the soluble plasma fraction, but is also expressed on different white blood cells (WBC) as well as platelets (12). The expression of PrP<sup>C</sup> on follicular dendritic cells in secondary lymphoid organs is required for prion transmission, especially during peripheral routes of inoculation (13-15). Experimental inoculations and subsequent passages in animal models, such as mice, hamsters, sheep, elk, and macaques, have shown variable levels of prion infectivity in tissues, such as spleen and muscle, as well as in plasma and buffy coat WBC (16). It has been estimated that the level of infectivity in blood is <100 infectious units/ml in mice inoculated with mouse-adapted human prions, magnitudes lower than that of the brain (17, 18).

Recently, several cases of prion transmissions have been reported in the United Kingdom. These patients contracted CJD years after having received non-leukodepleted blood products from asymptomatic donors who were later diagnosed with vCJD (19-22). Although the causal linkage between blood transfusion or factor VIII administration and these CJD cases cannot be proven experimentally, these incidences have ignited debates over the presence of prion infectivity in blood, and highlighted the urgent need to develop an antemortem blood test to identify presymptomatic CJD cases and to safeguard the blood supply. Several biological markers have been proposed, but the presence of PrP<sup>Sc</sup> remains the only reliable and specific indicator of prion diseases. Some progress has been made in the detection of prions in experimentally infected hamsters by protein misfolding cyclic amplification (PMCA), but its efficacy for human use has yet to be proven (23). Recently, some of us (J.G.S., C.D., M.D.G., B.L.M., S.B.P.) found that PrP<sup>Sc</sup> in the brains of sCJD patients binds to very-low density and low-density lipoproteins in plasma (24). Prions did not bind to high-density lipoproteins or other plasma components.
In this study, we established that effector memory T cells, of all the WBCs in circulation, express the highest number of surface PrP<sup>C</sup> per cell. Sporadic CJD patients, neurological controls, and healthy donors were found to have similar levels of surface PrP<sup>C</sup> on their WBC. We have isolated granulocytes, monocytes, B cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, natural killer (NK) cells, non-classical γδ T cells, and platelets from fresh whole blood samples from sCJD patients, neurological controls, and healthy donors, using magnets or fluorescence-activated cell sorting (FACS). Even after we optimized a conformation-dependent immunoassay (CDI) (25-27), we were unable to detect PrP<sup>Sc</sup> in any of the WBC fractions. Our results show that if prions are present in the blood of sCJD patients, they are present at low levels.

**MATERIALS & METHODS**

**Patients and blood samples**

Blood samples were obtained from 24 patients diagnosed with definite or probable sCJD. The diagnosis of definite sCJD was only made after immunohistological verification at autopsy. The cohort had a median age of 65 years and a median age of disease onset of 64. Among the 23 patients with known genotype, the frequencies of the PRNP codon 129 polymorphism were 52% M/M, 35% M/V, and 13% V/V (Table 1). Genetic prion diseases were ruled out by genomic DNA sequencing and postmortem analysis was performed on 18 of 23 deceased patients to confirm the diagnosis. For neurological controls, 27 age-matched subjects were identified. The majority (63%) of these controls were diagnosed with Alzheimer’s disease and the remaining patients suffered from other neurological disorders including progressive supranuclear palsy, corticobasal degeneration, frontal lobe dementia, dementia with Lewy bodies, and paraneoplastic limbic encephalitis. Whole blood samples from more than 30 anonymous healthy donors were obtained from the Stanford Blood Center. From each patient, 30-500 ml of blood was drawn and stored at -4 °C until processed.

**Blood sample processing**

Fresh whole blood samples were processed within 24 h of being drawn. Due to the scale of the WBC isolation procedures, we divided our blood sorting experiments into three phases, each tailored to maximize the yield of a different set of WBC. In Phase I that was used to isolate granulocytes, monocytes, CD4<sup>+</sup> T cells, and B cells, blood samples were centrifuged at low speed (162g, 10 min, 4 °C). The supernatant fractions were collected and centrifuged at higher speed (365g, 5 min, 4 °C). The pellets were collected, resuspended in PBS with 10 mM EDTA and the centrifugation repeated. The final pellet was resuspended in a FACS Lysing Solution (BD) to remove red blood cells, followed by a washing step in PBS with 10 mM EDTA. In Phases II and III that were used to isolate B cells, CD8<sup>+</sup> T cells, NK cells, γδ T cells, and platelets, whole blood samples were first concentrated by centrifugation at 4 °C for 10 min at 365g. The cell pellets were then reconstituted to twice the original blood volume in PBS with 10 M EDTA, and layered over a 60% isotonic Percoll gradient (GE Healthcare) for buffy coat isolation. Specimens for cell sorting were resuspended in PBS supplemented with 1% fetal calf serum, 0.25 U/ml DNaseI (Roche) and Complete protease inhibitors (Roche) before antibody staining.
**Flow cytometry and fluorescence microscopy**

All FACS was performed on the FACS Aria (BD) contained within a custom-made hood in our biosafety level 3 (BSL3) facility. Aerosol release from the Aria was monitored using fluorescent Glogerm particles (Glogerm, UT) \(^{28}\). Data were acquired using the FACS DiVa (BD) software and analyzed with the FlowJo software. WBC enumeration was performed using TruCOUNT tubes (BD) in combination with monoclonal antibodies against WBC markers CD3, CD4, CD8a, CD19, CD16, CD41, CD56, CD61, Vδ2TCR (BD). For fluorescent labeling of surface PrP molecules, we used monoclonal mouse antibodies 6H4 (Prionics) or 3F4, in conjunction with anti-mouse secondary antibodies conjugated to FITC (BD), PE (BD) or Alexa Fluor 594 (Molecular Probes). For isotype control, mouse IgG1 and IgG2a antibodies were used as the primary antibody. Alternatively, a panel of humanized anti-PrP Fab fragments, including D18, P, EST123 and EST149 \(^{29}\), was used with anti-human Fab secondary antibodies (Jackson Laboratory).

For quantitative measurement of surface PrP molecules, Percoll-processed buffy coats were stained first with 2.5 ug/ml of 6H4 or mouse IgG1 isotype control antibody for 1 h at 4 °C. At this saturating concentration, the 6H4 and control staining exhibited the greatest difference in their resulting fluorescence intensities. These samples were then stained with anti-mouse FITC antibody for 45 min at 4 °C and with a series of QIFIKIT calibration beads (DAKO) bearing known-amount, antibody-binding sites. In the final staining step, WBCs were labeled with different cell surface markers, as listed above. For comparison of naïve versus memory CD4^+ T cell, 6-color staining was performed using CD3-PE-Cy7, CD4-APC-Cy7, CD62L-PerCP-Cy5.5, CD45RA-APC, CD27-PE, and 6H4. Quantitative flow cytometry on platelets was performed on magnetically isolated platelet fractions (see below) and gated according to size and granularity. Each time quantitative FACS analysis was performed, a standard curve was generated by the aforementioned calibration beads, correlating fluorescence intensity with the number of bound antibodies. The number of PrP molecules per cell was extrapolated from this calibration, upon subtracting the background fluorescence (isotype control) from the total fluorescence (6H4). All determinations of surface PrP molecules were performed in duplicate.

For fluorescence microscopy of peripheral NK cells, circulating CD4^- lymphocytes were first isolated from lysed whole blood using FACS and then CD3-depleted with Dynal beads. These cells were labeled with the anti-PrP 3F4 antibody and Alexa Fluor 594-conjugated anti-mouse secondary antibody (Molecular Probes), followed by anti-CD56 (N-CAM) FITC (BD).

**FACS sorting of WBC**

Blood samples were FACS sorted using the FACS Aria (BD) under a custom 4-way-sort mode to ensure maximal separation efficiency as well as purity. Routine decontamination was performed using 1 M NaOH, in order to avoid potential crossover contamination between samples. Because the BD FACS Aria can only isolate a maximum of four cell populations at a time, we isolated different sets of circulating WBCs in two phases. A third phase to isolate platelets was performed using magnetic activated cell sorting (MACS; see below.)

*Lab Invest. Author manuscript; available in PMC 2009 December 02.*
For Phase I isolation, WBCs were stained with CD4-PE and CD19-APC to isolate granulocytes [forward scatter (FSC) high, side scatter (SSC) high, CD4+, CD19+], monocytes (SSC high, CD4+, CD19+), CD4+ T cells (SSC low, CD4++, CD19+) and B cells (SSC low, CD19+, CD4+). For isolation of WBC subpopulations in Phase II,uffy coats were stained with CD3-PE-Cy5, CD8-APC-Cy7, CD19-APC, CD56-FITC and Vδ2TCR-PE, to enable concomitant 5-color sorting of CD8+ T cells (CD3+, CD8+, Vδ2 TCR+, CD19+), NK cells (CD3+, CD8+, CD56+, Vδ2 TCR+), non-classical γδT cells (CD3+, CD8+, Vδ2 TCR+, CD19+) and B cells (CD19+, CD3+, CD56+, Vδ2 TCR+). The purity of all post-sort samples was confirmed by FACS before the cells were centrifuged at 365g for 30 min, and frozen in aliquots.

**Magnetic sorting of platelets**

For isolation of platelets, Percoll-processed buffy coats were stained with anti-CD61 antibody-coated MACS microbeads (Miltenyi Biotec), before fractionation by the AutoMACS machine (Miltenyi Biotec). In order to eliminate cross-contamination, the AutoMACS was decontaminated using 10% bleach between samples and all AutoMACS LS columns (Miltenyi Biotec) were limited to single use.

**Conformation-dependent immunoassay (CDI)**

The CDI was performed as described previously (27), with several modifications. First, purified WBC were resuspended in a PBS, pH 7.4, containing 2% (w/v) Sarkosyl, 5 mM ε-aminocaproic acid (6-aminohexanoic acid, EACA, Sigma), 1 mM MgCl₂, 1% (w/v) brain homogenate from Prnp<sup>0/0</sup> mice, 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), and 1 μg/ml each of aprotinin and leupeptin (Sigma). The cells were homogenized by one 75-s cycle in a reciprocal homogenizer Mini-BeadBeater-8 (BioSpec Products, Inc., OH) and treated with 50 IU/ml of benzonase for 30 min at 37 °C. PrP<sub>Sc</sub> was precipitated with 0.32% (w/v) PTA for 1 h at 37 °C, then centrifuged at 14,000 g for 30 min. The pellet was resuspended in 100 μl of PBS, pH 7.4, containing 0.2% Sarkosyl, 0.1 mM PMSF, and 1 μg/ml each of aprotinin and leupeptin, then split into two aliquots. One aliquot, denoted D for denatured, was denatured at 80 °C in 4 M guanidinium HCl; the other aliquot was left undenatured and designated N for native. Aliquots of 20 μl from each fraction were directly loaded into wells of white Greiner strip plates (Greiner Bio-One) prefilled with 200 μl of assay buffer (Wallac) and coated previously with MAR1 anti-PrP capture antibody (30). The captured PrP was detected by a europium-conjugated anti-PrP mAb 3F4 (31) or recombinant antibody fragment (recFab) P (26). The amount of PrP<sub>Sc</sub> in a given WBC sample is directly proportional to the difference in the fluorescence signals (measured in counts per minute) from denatured and native samples, or (D-N).

The preparation and purification of recHuPrP(90-231) used as a calibrant in the CDI were described previously (24, 32). The purity of recHuPrP(90-231) was assessed by mass spectrometry. The molecular mass for recHuPrP(90-231) was 16,059 daltons, as expected. The protein concentration was determined by UV spectroscopy using the predicted molar extinction coefficient of 24420 M<sup>-1</sup>. The purified protein was dissolved in 4 M GdnHCl and 50% Stabilcoat (SurModics, Eden Prairie, MN), and stored at -80 °C.
The concentration of total PrPC in fractionated WBC was calculated from the CDI signal of non-denatured cell lysate. Proteinase K treatment of WBC samples decreased the signal/noise ratio and was therefore excluded from this protocol.

**Statistical analysis**

Dot plots were produced using the KaleidaGraph software and mean values shown as horizontal lines. In comparing different patient groups, $p$ values were calculated using type 2 Student's $t$ test.

**RESULTS**

**PrP**<sup>C</sup> expression on human WBC

PrP<sup>C</sup> expression is required for prion replication (33, 34). Previously, surface PrP<sup>C</sup> was detected on all the major subsets of circulating WBCs in humans, albeit at levels much lower than those found in the CNS (12, 35). Because the expression pattern of PrP<sup>C</sup> may reflect the capacity of different WBC to support prion replication, we performed detailed FACS analyses to identify all of the WBC subpopulations in fresh human peripheral blood. PrP<sup>C</sup> was found on the surfaces of granulocytes, monocytes, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, non-classical γδT cells, CD19<sup>+</sup> B cells, CD56<sup>+</sup>CD3<sup>-</sup> NK cells, and CD41<sup>+</sup>CD61<sup>+</sup> platelets (Fig. 1A; data not shown).

On the surface of neurons, PrP<sup>C</sup> was found to interact with the neural cell adhesion molecule (N-CAM) and subsequently activate p59<sup>fyn</sup> mediated signaling (36-39). Because NK cells coexpress PrP<sup>C</sup> and N-CAM, we performed immunostaining and fluorescence microscopy to visualize their expression pattern. Circulating CD3<sup>-</sup>CD4<sup>-</sup> lymphocytes were purified by FACS and MACS sorting. The NK cells in this fraction were stained first with anti-PrP 3F4 antibody and Alexa Fluor 594-labeled anti-mouse secondary antibody, and then with anti-CD56 (N-CAM) FITC. On unpolarized cells, PrP<sup>C</sup> (red) assumes a punctate appearance, evenly distributed over much of the surface (Fig. 1B, top panels). N-CAM (green) does not localize with PrP<sup>C</sup> in unpolarized cells. However, on polarized NK cells in the same fraction, PrP<sup>C</sup> and N-CAM colocalized at one end of the cell, possibly due to redistribution of lipid rafts and the associated GPI-anchored proteins (Fig. 1B, bottom panels).

**Factors affecting PrP<sup>C</sup> expression in WBCs**

The N-terminus of PrP<sup>C</sup> in hamster brain is sensitive to cleavage by proteases (40). We therefore tested whether the duration of sample storage affected WBC-associated PrP<sup>C</sup>. Using a panel of anti-PrP Fab fragments recognizing different linear epitopes along the PrP polypeptide, we monitored changes in the levels of PrP<sup>C</sup> on the surface of WBCs, as processed human buffy coats were allowed to age at 4 °C (Fig. 1C). In fresh samples less than 24 h old, each anti-PrP Fab registered a different maximum staining capacity, which we normalized to 100% for comparison. With samples stored for 2 days, antibody binding was specifically reduced for Fab fragments E123 and E149 that target the N-terminus of PrP<sup>C</sup> (Fig. 1C), indicating that WBC-associated PrP<sup>C</sup> molecules were subjected to limited proteolysis. When experiments were performed in the presence of protease inhibitors, Fabs E123 and E149 showed increased binding to the aged samples. Because sample storage
seemed to affect antibody detection of PrP\(^C\), all specimens were analyzed immediately after processing and only fresh human peripheral WBC less than 24 h old were used.

To study the influence of cell differentiation, we used QIFIKIT calibration beads (DAKO) in conjunction with six different WBC surface markers to quantify the number of cell-associated PrP\(^C\) molecules in four human buffy coats (Fig. 2A). Of the three types of CD4\(^+\) T cells (T4), CD45RA\(^-\)CD62L\(^-\) effector memory (EM) cells had the highest level of PrP\(^C\) expression (12854 ± 1695), followed by CD45RA\(^-\)CD62L\(^+\)CD27\(^+\) central memory (CM) cells (7867 ± 287) and CD45RA\(^+\)CD62L\(^+\)CD27\(^+\) naïve (N) cells (4944 ± 589). The differences in PrP\(^C\) levels were statistically significant between all three sets of T4 cells (N v. CM: \(p=0.01\); N v. EM: \(p=0.004\); CM v. EM: \(p=0.04\); Fig. 2B). Because the relative abundance of these T cell populations can change dramatically during viral infections, a patient's immunological status can modulate the levels of PrP\(^C\) molecules in circulation.

**Surface PrP on WBCs and platelets**

We determined whether the amount of total surface PrP\(^C\) on WBC differs in patients with sCJD compared to healthy donors, using quantitative FACS analysis. Fresh buffy coats from sCJD patients, age-matched controls with other neurological diseases, and anonymous healthy blood donors (Table 1) were stained in duplicate with anti-PrP antibody 6H4 and IgG1 isotype control, along with a series of calibration beads from the QIFIKIT kit (DAKO). To obtain the number of PrP\(^C\) molecules per cell, the number of bound isotype control antibody was subtracted from the number of bound 6H4. To identify different WBC populations, buffy coats were also labeled with a combination of cell markers: CD3\(^+\)CD4\(^+\)CD8\(^-\) for CD4 T cells, CD3\(^+\)CD8\(^+\)CD4\(^-\) for CD8 T cells, CD3\(^-\)CD56\(^+\)CD4\(^-\) for NK cells, and CD41\(^+\)CD61\(^+\) for platelets. All of the three types of lymphocytes expressed between 5000 and 10,000 PrP\(^C\) molecules on their surfaces, whereas only low levels of PrP\(^C\) were found on platelets (Fig. 3). It is notable that a small fraction of platelets (<14%) was found to express high levels of PrP\(^C\) [172,806 ± 25,504 (n = 10); data not shown]. Because the significance of these novel, high-PrP\(^C\)-expressing platelets is unknown and their frequency varied widely among samples, they were excluded from further analysis. The sCJD patients expressed less PrP\(^C\) on their platelets than healthy donors (\(p=0.05\)), as did age-matched, neurological controls (\(p=0.02\)). No significant differences were found between sCJD patients and neurological controls in any of the WBC populations tested (Fig. 3).

In order to verify the differential expression of PrP\(^C\) on lymphocytes and platelets, we sorted by FACS six different cell types from five healthy donors and quantified their total PrP\(^C\) content using the CDI (Fig. 4). One million cells per specimen were assayed for all cell types, except platelets, which were tested at four different concentrations. CD4\(^+\) and CD8\(^+\) T lymphocytes expressed more PrP\(^C\) per cell than platelets, which showed a PrP\(^C\) expression level similar to that of B cells, granulocytes, and monocytes (Fig. 4). Nonetheless, the majority of cell-associated PrP\(^C\) is attributed to the platelet fraction because of the overwhelming abundance of platelets in circulation compared to other WBC fractions.
WBC profile of sCJD patients

We next examined the composition of circulating WBCs in sCJD patients. Five to eight fresh whole blood samples were collected per control and patient group, and analyzed with TruCOUNT beads (BD Biosciences) and WBC markers (Table 2). As expected, platelets were the most abundant cell species in peripheral blood, followed by granulocytes and monocytes. In six of seven subtypes tested, the frequencies of circulating WBCs were similar for all three patient groups. Granulocytes were more abundant in sCJD samples compared to healthy donors ($p=0.07$; Fig. 5). However, due to considerable overlap between the data sets from sCJD patients and neurological controls, sCJD cases could not be distinguished by their elevated granulocyte count alone. The blood of sCJD patients also contained more CD41+CD61+ platelets than samples from both healthy donors and neurological controls (Table 2). These trends, however, were not statistically significant until outliers were omitted from the analysis ($p=0.06, 0.08$).

Further fractionation of WBCs

We fractionated all major WBC subpopulations from healthy donors, neurological controls and symptomatic sCJD patients. Purified cells were then frozen in aliquots until subsequent CDI testing.

First, we concentrated whole blood samples by centrifugation and isolated the most abundant nucleated WBCs in circulation, namely granulocytes, monocytes, CD4+ T cells and B cells (Supplementary Fig. S1). This group of target cells possesses a diverse range of biological and physical characteristics, such as function, half-life in circulation, and trafficking. Using two scattering parameters and surface markers CD4 and CD19, we successfully sorted these cells to >95% purity (Supplementary Fig. S1). Next, B cells, CD8+ T cells, NK cells, and γδ T cells were isolated (Supplementary Fig. S2). Being relatively rare in blood, these lymphocytes normally have a circulating frequency between 1 to 0.1%, which could limit our sorting efficiency. Therefore, we enriched samples for lymphocytes prior to FACS sorting, by processing the whole blood samples into buffy coats using Percoll gradient centrifugation. The WBCs were then fractionated in a 5-color sorting scheme, using surface markers CD3, CD8, CD19, CD56 and γδ TCR, as well as physical parameters (Supplementary Fig. S2). An average purity of >95% was achieved for B cells, CD8+ T cells, and NK cells. Non-classical γδ T cells were only enriched to ~70%, possibly due to their extremely low starting frequency as well as to variability in the labeling of the γδ TCR marker. Finally, platelets were isolated using CD61 MACS beads (Miltenyi Biotec) and the AutoMACS system (Miltenyi Biotec) (Supplementary Fig. S3).

CDI detection of PrPSc in WBC and platelets

In order to measure the concentration of PrPSc in WBCs using the CDI, samples were assayed in duplicate using two different Europium-labeled antibodies, 3F4 and HuM-P, for detection. Sorted CD4+ T cells, B cells, monocytes, and granulocytes from 5 subjects in each patient group were pooled and tested initially by the standard CDI protocol. We found no positive PrPSc signals in these WBC populations (data not shown). Similarly, repeating this experiment using 10x as many cells per sample generated no PrPSc signal above background. After subsequent optimization, we tested B cells, granulocytes, NK cells,
platelets, CD8+ cells, and γδ TCR T cells purified from individual patient samples; ~10 specimens from each group were used. The optimized CDI was able to detect PrPSc in Prnp0/0 brain homogenate spiked with 0.05% brain homogenate over a 10,000-fold range of brain homogenate concentrations (Fig. 6). The detection limit of recHuPrP(90-231) was 0.5 pg/ml, equivalent to ~6 attomoles per plate well. The CDI signal and calculated concentrations of PrPSc fluctuate in all groups around the detection limit of the CDI. None of the purified WBC fractions from sCJD patients exceeded the background levels of donor or neurological control groups (Fig. 6). Cell densities per sample were: 5 × 10^5 B cells, 10^6 granulocytes, 5 × 10^5 NK cells, 3 × 10^7 platelets, 5 × 10^5 CD8+ T cells, 5 × 10^5 CD4+ T cells, and 5 × 10^5 monocytes.

**DISCUSSION**

In studies described here, we found that PrPC is widely distributed among subpopulations of circulating WBCs. We determined that the highest levels of PrPC were found in a subpopulation of T lymphocytes: ~12,000 PrPC molecules were found per CD4+CD45RA-CD62L-effector memory T helper cell. Although platelets expressed low levels of PrPC on their surface, their high abundance in circulation resulted in the majority of PrPC being platelet-associated.

The presence of PrPC on the surface of peripheral blood cells has been reported previously (12, 35, 41, 42). In fact, PrPC is also known as CD230, according to the immunological nomenclature of cluster differentiation markers (43). Nonetheless, more than 20 years after the discovery of PrPC, its biological function remains unclear. The coexpression of PrPC and N-CAM as well as their likely physical interactions on the surface of neurons suggest a role in signal transduction (36-39). This is consistent with our finding that these two proteins colocalize in polarized NK cells (Fig. 1B). On the physiological level, the elevated expression of PrPC on effector memory T cells compared to central memory T cells and naïve T cells (Fig. 2) suggests an involvement in WBC homing and/or extravasation, which has recently been demonstrated in vitro for monocytes (44).

In contrast to an earlier report (45), we were able to detect low levels of PrPC expression using the sandwich CDI on purified granulocytes, monocytes, CD4+ T cells, CD8+ T cells, B cells, and platelets (Fig. 4). This ubiquitous expression of PrPC on circulating human WBCs contrasts with our observations in wild-type, inbred FVB and CD-1 mice. Using a panel of seven anti-PrP Fab fragments, we were able to detect surface expression of mouse PrPC on LPS-stimulated splenocytes, bone marrow-derived dendritic cells and mast cells, but not on circulating CD4+ T cells, CD8+ T cells, NK cells, B cells, monocytes or granulocytes (data not shown). However, transgenic mice overexpressing truncated PrPC, denoted Tg9949 mice, have readily detectable PrPC on their peripheral lymphocytes (data not shown). Because lymphocytes upregulate PrPC upon activation, the lack of surface PrPC in peripheral WBCs in wild-type mice is likely to reflect the naïve immunological status of these captive laboratory animals. Similarly, we found fewer PrPC molecules on the cell surface of circulating naïve T cells compared to memory T cells in human blood (Fig. 3).
Mononucleated WBCs such as T cells and NK cells generally express at least 10 times more PrPC than platelets, as determined by quantitative FACS (Fig. 3) and the CDI (Fig. 4). This observation is consistent with a previous study that used a direct FITC conjugate of the anti-PrP antibody, 6H4 (42). Others using the same QIFIKIT reagent for quantitative FACS obtained slightly different results, which could be due to their use of another anti-PrP antibody (3F4) and/or unstained samples as negative controls (35).

**PrP expression in sCJD patients**

Using quantitative FACS analysis, we found that neither WBC composition nor the amount of cell-surface PrPC molecules was altered in patients dying of sCJD. Eight different WBC fractions types from the peripheral blood of sCJD patients were assessed for PrPSc. We were unable to find any evidence for PrPSc in purified granulocytes, monocytes, B cells, CD4+ T cells, CD8+ T cells, NK cells, non-classical γδT cells, or platelets.

Although we observed slightly lower levels of PrP on the platelets of sCJD patients and neurological controls compared to healthy donors (p=0.02 and 0.05, respectively; Fig. 3) (45, 46), we hesitate to assign importance to this finding for several reasons. First, our sample size was small (n=3-5) and second, the differences were only statistically significant after a small subpopulation of platelets with an uncharacteristically high level of PrP (172,806 ± 25,504) was excluded from the analysis (data not shown). Third, because platelet PrP was reduced in both sCJD patients and neurological controls, it cannot be used as a specific biological marker for prion disease. In half of our sCJD patients, the frequency of circulating granulocytes was increased compared to healthy donors (p=0.07; Table 2, Fig. 5). This finding might be a consequence of secondary infections that often occur in debilitated patients.

Eight subpopulations of WBCs were purified from the peripheral blood of over 100 individuals. We were unable to detect PrPSc in platelets, NK cells, CD8+ T cells, CD4+ T cells, B cells, monocytes or granulocytes, using the CDI. The concentration of PrPSc in 0.05% sCJD brain homogenate measured by CDI is ~7 ng/ml (Fig. 6), with a detection limit of ~0.5 pg/ml. Because the concentration of prions in 0.05% sCJD BH is 10^3.0-10^4.3 infectious units (IU) per ml (27), we estimate that the limit of prion detection in this CDI protocol is ~1.4 IU per ml or less. Thus, our negative findings are consistent with the inability of retrospective epidemiological investigations to identify sCJD cases associated with blood transfusions (47). Importantly, leukodepletion in experimental scrapie studies did not completely eliminate prion infectivity in blood (48) and transfusions of blood from sheep with scrapie or from deer with CWD efficiently transmitted disease to recipient animals (49-51). The high-affinity binding of PrPSc to low density lipoproteins (LDL) (24) raised the possibility that prions in human blood are primarily found in the plasma fraction. Whether low levels of prions can be detected in the plasma lipoproteins of sCJD patients using CDI and bioassay in mice expressing chimeric Hu/Mo PrP transgenes (52) remains to be determined (J. Safar and S. B. Prusiner, in preparation).

**Supplementary Material**

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*Lab Invest. Author manuscript; available in PMC 2009 December 02.*
ACKNOWLEDGMENTS

The authors thank Chris Roden, Kristen Pomroy, Kristen Schardein-Fox, and Amy Kuo for their technical assistance; Ingrid Schmid (UCLA), Marty Bigos (The Gladstone Institute) and all the flow cytometry engineers, especially Romulo Castrence (BD), for their assistance in the installation and maintenance of our BSL3 FACS Aria within a biosafety hood; and to Hang Nguyen for her skillful editorial assistance.

Support: NIH grants AG02132, AG10770, and AG021601; U.S. Department of Defense contract.

Abbreviations

CDI  conformation-dependent immunoassay
FACS  fluorescence-activated cell sorting
N-CAM  neural cell adhesion molecule
NK  natural killer
PMCA  protein misfolding cyclic amplification
PrP  prion protein
PrPC  cellular isoform
PrPSc  disease-causing isoform
PTA  phosphotungstic acid
sCJD  sporadic Creutzfeldt-Jakob disease
SSC  side scatter
vCJD  variant Creutzfeldt-Jakob disease
WBC  white blood cells

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Fig. 1.
Surface expression of PrP<sup>C</sup> on human peripheral white blood cells (WBC). (A) FACS analysis of anti-PrP<sup>C</sup> antibody 3F4 staining on CD4<sup>+</sup> T cells (left), CD8<sup>+</sup> T cells (middle), and CD19<sup>+</sup> B cells (right). Staining with 3F4 is shown as a solid black line, with IgG2α isotype control shown in gray. (B) Distribution of surface PrP<sup>C</sup> (red) and CD56/NCAM (green) molecules on NK cells. On the majority of NK cells, PrP<sup>C</sup> had a punctate expression pattern that did not correspond to that of CD56. However, PrP<sup>C</sup> and CD56 colocalized in polarized cells (right panels). (C) N-terminal proteolysis of surface PrP<sup>C</sup> on WBC. Fresh
buffy coats were resuspended in RPMI-1640 medium in the presence or absence of protease inhibitors cocktails for 2 days. The integrity of cell-associated PrPC molecules was then probed with a panel of anti-PrP Fab fragments, including E123 and E149 that bind to the N-terminal portion of the PrP molecule. Results shown are representative of two independent experiments.
Fig. 2. Surface expression of PrP\(^C\) on peripheral CD4\(^+\) T cells at different stages of differentiation (A). Upon 6-color FACS analysis, CD3\(^+\)CD4\(^+\) T cells (T4) were phenotyped as naïve (N; CD45RA\(^+\)CD62L\(^+\)CD27\(^+\)), central memory (CM; CD45RA\(^-\)CD62L\(^+\)CD27\(^+\)), and effector memory (EM; CD45RA\(^-\)CD62L\(^-\)) cells. FACS staining with anti-PrP\(^C\) Fab fragment 6H4 is shown as a solid black line, with isotype IgG1 control in gray. (B) Histograms show that both types of antigen-experienced T4 cells expressed significantly higher levels of surface PrP\(^C\) than naïve T4 cells. The difference between EM and naïve cells was more than 2x. \(P\) values were determined by Student’s t test. Specimens from at least four donors were used in this analysis. All samples were tested in duplicate.
Fig. 3.
Surface PrP<sup>C</sup> expression on circulating WBCs was similar in sCJD patients and controls. Quantification of surface PrP molecules on circulating WBC subpopulations by quantitative FACS. Freshly prepared buffy coats were stained with anti-PrP antibody 6H4 and analyzed with QIFIKIT (DAKO) reagent. Negative control samples were concomitantly stained with isotype control IgG1 antibody. The absolute number of surface PrP molecules per cell was obtained by subtracting the background value from the total number of bound 6H4 molecules. The CD41<sup>+</sup>CD61<sup>+</sup> platelets in our specimens produced two distinct populations of PrP<sup>C</sup> expression; only the predominant platelet population expressing low levels of PrP<sup>C</sup> was included in this analysis. The statistical differences in PrP<sup>C</sup> expression between sCJD patients, neurological controls, and healthy donors were calculated by Student's t test. Mean numbers of PrP<sup>C</sup> molecules per cell, for each patient group and each WBC subpopulation, are indicated by the horizontal lines. Each data point represents a unique patient. All samples were tested in duplicate.
Fig. 4. T4 and T8 cells express more PrP\textsuperscript{C} than the other cell subtypes. Concentration of PrP\textsuperscript{C} in FACS-sorted peripheral WBCs from five healthy donors was compared by sandwich CDI. B cells (B), granulocytes (Gr), monocytes (Mo), platelets (PLT), CD4\textsuperscript{+} T cells (T4), and CD8\textsuperscript{+} T cells (T8). All samples were tested in duplicate using MAR1 antibody for capture, 0.1 μg/ml of Europium-labeled 3F4 antibody for detection, and recHuPrP(90-231) as a calibrant. Platelets were assayed at four different concentrations.
Fig. 5.
Levels of circulating granulocytes in sCJD patients (circles), neurological controls (squares), and healthy donors (triangles). Each symbol represents an individual sample and the average of each group is indicated by a horizontal line. Each group consisted of five to eight patient samples. All specimens were tested in duplicate. *P* values were calculated by Student’s *t* test.
CDI testing for PrP\textsuperscript{Sc} in individual patient samples. Sorted cell subtypes from sCJD patients (circles), healthy donors (diamonds), and neurological controls (triangles) were assayed in the CDI for the presence of PrP\textsuperscript{Sc}. Results were similar for all samples in all patient groups. Number of cells per specimen: 5 × 10\textsuperscript{5} B cells (B); 1 × 10\textsuperscript{6} granulocytes (Gr); 5 × 10\textsuperscript{5} NK cells (NK); 3 × 10\textsuperscript{7} platelets (PLT); 5 × 10\textsuperscript{5} CD8\textsuperscript{+} T cells; 2 × 10\textsuperscript{5} non-classical γδ T cells. Similar results were obtained using 3 × 10\textsuperscript{8} platelets, 5 × 10\textsuperscript{5} monocytes, and 5 × 10\textsuperscript{5} CD4\textsuperscript{+} T cells (data not shown). Each symbol represents the measurement from an individual sample; the horizontal line indicates the mean level for each group and cell type. All specimens were assayed in duplicate. B cells, platelets, and non-classical γδ T cells were tested twice. Each plate contained serial dilutions of recHuPrP(90-231) and sCJD brain homogenate (BH) diluted to 0.05% (w/v) as a positive control.
| Groups                  | Patient composition        | Median age (range) | Median age of onset (range) | Female/Male | PRNP codon 129 |
|------------------------|---------------------------|--------------------|-----------------------------|-------------|----------------|
| sCJD (n=24)            | 75% pathology confirmed  | 65 (49-79)         | 64 (49-78)                  | 10/14       | M/M            |
|                        |                           |                    |                             |             | M/V            |
|                        |                           |                    |                             |             | V/V            |
| Neurological controls  | 63% probable/Alzheimer’s Disease | 66 (49-83) | 61 (46-78) | 17/10       | M/M            |
| (n=27)                 |                           |                    |                             |             | M/V            |
|                        |                           |                    |                             |             | V/V            |

Table 1

Demographics of the cohorts of sCJD patients and neurological controls used in this study.
Table 2
Relative abundance of WBC components of fresh whole blood from sCJD patients and controls.¹

| WBC component | Healthy donors (million/ml ± SEM) | Neurologic controls (million/ml ± SEM) | sCJD (million/ml ± SEM) |
|---------------|-----------------------------------|----------------------------------------|------------------------|
| Gr            | 2.13 ± 0.24                       | 3.83 ± 0.30                            | 5.49 ± 1.88            |
| T4            | 0.52 ± 0.13                       | 0.98 ± 0.18                            | 0.77 ± 0.16            |
| T8            | 0.25 ± 0.04                       | 0.34 ± 0.06                            | 0.31 ± 0.09            |
| B             | 0.26 ± 0.09                       | 0.18 ± 0.04                            | 0.22 ± 0.04            |
| NK            | 0.30 ± 0.06                       | 0.52 ± 0.13                            | 0.41 ± 0.05            |
| Mo            | 0.45 ± 0.06                       | 0.50 ± 0.05                            | 0.60 ± 0.10            |
| PLT²          | 143 ± 12                           | 138 ± 20                                | 221 ± 42               |

¹ Each patient group consisted of 5 to 8 individuals. All samples were tested in duplicate.

² Platelets were gated on CD41⁺CD61⁺ double-positive subpopulation