original paper

haematologica 2001; 86:146-153
http://www.haematologica.it/2001_02/0146.htm

Cellular prion protein is expressed on peripheral blood mononuclear cells but not platelets of normal and scrapie-infected sheep

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Background and Objectives. Transmissible spongiform encephalopathies (TSEs) including sheep scrapie are characterized by the conversion of a normal, cellular prion protein (PrP^{c}) to an abnormal protease-resistant form (PrP^{Sc}). Like human peripheral blood, the peripheral blood of scrapie-infected sheep remains one possible source of disease transmission. As a first step in understanding the disease requirements in the natural scrapie host, the presence of PrP^{c} was evaluated in peripheral blood cells from five normal and five scrapie-infected Suffolk sheep.

Design and Methods. Live peripheral blood cells from normal and scrapie-infected sheep were analyzed for the presence of PrP using flow cytometry and reverse transcriptase-polymerase chain reaction (RT-PCR).

Results. PrP mRNA was detected in peripheral blood mononuclear cells (PBMC) but not in platelets or granulocytes. Consistent with PrP mRNA expression, cell-surface expressed PrP was detected on PBMC, but was not detected on granulocytes, platelets, or erythrocytes. Two-color flow cytometric analysis of PBMC specific phenotypes revealed that regardless of scrapie-status, expression of PrP was significantly higher on B2 positive B-lymphocytes than on CD4, CD8, WC1 positive T-lymphocytes or CD14 positive monocytes. In addition, PrP expressed on PBMC from normal and scrapie-infected sheep was sensitive to proteinase K (PK) and phosphatidylinositol-specific phospholipase C (PIPLC).

Interpretation and Conclusions. Regardless of the scrapie-status of the sheep, resting PBMC transcribe Pr^{Pc} and express Pr^{Pc} as a cell-surface protein sensitive to both PK and PIPLC. Because of the abundance of Pr^{Pc} on PBMC, future diagnostic tests using PK and PIPLC to discriminate between protease sensitive and resistant PrP must be carefully evaluated.

Key words: prion, transmissible spongiform encephalopathy (TSE), blood, flow cytometry, RT-PCR

Sheep scrapie is part of a group of fatal neurodegenerative diseases called transmissible spongiform encephalopathies (TSEs) or prion diseases. These diseases are characterized by the accumulation of PrP^{Sc} in sheep scrapie, PrP^{BSE} in bovine spongiform encephalopathy (BSE), PrP^{CWD} in chronic wasting disease (CWD), PrP^{CJD} in Creutzfeldt-Jacob disease (CJD), and PrP^{nvCJD} in new variant CJD. Since PrP^{Sc} and infectivity co-purify,[1] the presence of PrP^{Sc} is considered a marker for TSEs. Prion diseases are caused by the post-translational modification of a normal cellular host-derived protease-sensitive prion protein, PrP^{c}, to an abnormal protease-resistant prion protein, PrP^{Sc}.[2] PrP^{c} is required for spread of disease in the central nervous system.[3-5] In addition, PrP^{c} is required for PrP^{Sc} accumulation on specific cell types comprising the lymphoid system. In mice studies, the presence of PrP^{c} on follicular dendritic cells but not peripheral B- and T-lymphocytes is required for PrP^{Sc} accumulation in lymphoid tissues and clinical disease of mice.[6-8] And, B-lymphocytes are essential for progression of disease.[7] Although mice studies offer valuable information on lymphoid requirements for disease, little information is known regarding lymphoid cell requirements for disease or the location of PrP^{c} in the lymphoid system in the natural scrapie host, sheep.

Because of the PrP^{c} requirement for disease and the concern of PrP^{nvCJD} transmission from peripheral blood, the presence of PrP^{c} in human peripheral blood has been previously investigated. PrP mRNA and PrP^{c} were detected in human peripheral lymphocytes and were detected at a lower level in erythrocytes and granulocytes.[9] In addition, PBMC expressed higher amounts of PrP^{c} than granulocytes during differentiation.[10] In human platelets, PrP mRNA and secreted and cell-associated PrP^{c} were detected.[11,12] More recently, cell-surface PrP^{c} was detected and quantified on B-lymphocytes, T-lymphocytes, monocytes, and platelets in human peripheral blood.[12] Although PrP mRNA and PrP^{c} have been detected in human peripheral blood, the detection of PrP^{nvCJD} in peripheral blood has not been reported.

Like human peripheral blood, the peripheral blood of scrapie-infected sheep remains one possible source of disease transmission. Although studies using fluorescent-labeled PrP peptides and capillary electrophoresis have suggested the presence of PrP^{Sc} in peripheral blood leukocytes from scrapie-infected sheep,[13] disease transmission from the peripheral blood of scrapie-infected sheep has not been demonstrated.[14] As a first step in understanding the disease requirements in the natural scrapie host, the presence of PrP was evaluated in peripheral blood cells from five normal and five scrapie-infected Suffolk sheep.

Design and Methods

Animals

Venous peripheral blood from five 3-year old normal Suffolk ewes and five 2-3 year old scrapie-infected ewes was drawn by jugular puncture into [10 mM EDTA]$_f$ coated syringes or vacutainer tubes. Complete blood count (CBC) differentials were determined at the WSU veterinary medicine clinical pathology laboratory. Normal and scrapie-infected ewes were genotyped with QQ at 171 in the amino acid sequence. Normal sheep used in this study are defined as having no previous exposure to scrapie and no detectable PrP^{Sc} accumulation in the lymphoid tissue of the third eyelid whereas scrapie-infected sheep are defined as having PrP^{Sc} accumulation in the lymphoid tissue of the third eyelid.[15,16] The scrapie-infected sheep used in this study have natural field scrapie.

Preparation and sorting of peripheral blood cells for RNA isolation

Leukocytes with platelets were isolated from peripheral whole blood using RBC lysis solution (Puregene) according to manufacturer's instructions. PBMC were isolated by gradient purification from whole peripheral blood using previously described methods[17] with Accu-Paque (density=1.086, Accurate Chemical and Scientific Corp.). Leukocytes with platelets and PBMC were washed three times using 1 × PBS/10 mM EDTA pH 6.9 (made in DEPC-treated water). Cells were counted and cell viability and morphology were checked on a Neubauer hemacytometer in 0.2% trypan blue. Leukocytes with platelets were sorted by size into platelets, peripheral blood mononuclear cells (PBMC) and basophils, and neutrophils and eosinophils using side (SSC) and forward scatter (FSC) on a FacsVantage SE flow cytometer equipped with a 488nm argon laser (Becton-Dickinson). For each gated cell population, 10^6 sorted cells were collected. The cells were centrifuged at 2,060 × g for10 minutes at 4°C. Sorted cells were confirmed morphologically, and flow cytometric cell counts were confirmed using a Neubauer hemacytometer. A remaining 10^6 non-sorted leukocytes were analyzed in addition. One microliter of glycogen at 20 mg/mL (Roche) followed by 0.75 mL of Trizol-LS (Life Technologies) was added to the sorted and non-sorted cells and placed at –20°C. Total RNA isolations and RT-PCR were performed (see below).

Gradient purified PBMC were incubated on ice for 15-30 min with the following mouse monoclonal antibodies (mAbs) at 0.75 μg per 1×10^6 PBMC per 50 μL first wash buffer (FWB): 17D1 (IgG_1),[18] CACT80C (IgG_1),[19] and BAQ4A (IgG_1)[20] to identify T-lymphocytes and the mAbs PIg45A2 (IgG_{2b})[21] and BAQ44A (IgM)[22] to identify B-lymphocytes. Three washes (using 2-3 times the original cell volume) with FWB (1×PBS pH 7.2, 10% acid citrate dextrose, 0.1% NaN_3, 2% γ-globulin free horse serum and 1% phenol red solution) were performed by centrifuging at 500×g for 5 min at 4°C, discarding the supernatant, and adding FWB. PBMC were incubated on ice in the dark for 15-30 minutes with 50 μL of the following secondary antibodies per 1×10^6 PBMC diluted in FWB: fluorescein (FITC)-conjugated goat anti-mouse IgG_1 1:100 (Caltag) and phycoerythrin (PE)-conjugated goat anti-mouse IgM 1:200 (Caltag) and IgG_2b 1:100 (Caltag). Two washes were performed as described above using second wash buffer (SWB) (same recipe as FWB but without γ-globulin free horse serum). PBMC were resuspended into 1× PBS/10mM EDTA pH6.9 and were gated based on size using SSC and FSC, and the T-lymphocytes, B-lymphocytes, and remaining unseparated PBMC populations were gated based on fluorescence phenotype using a FACsVantage flow cytometer. Then 10^6 T-lymphocytes, B-lymphocytes, and unseparated PBMC were collected by centrifuging at 1,000 × g for 10 min at 4°C, and PBMC resuspended in the residual liquid. One microliter of glycogen 20 mg/mL followed by 0.75 mL of Trizol-LS was added to the sorted cells, and 10^6 non-sorted cells. These were placed at –20°C for total RNA isolations and RT-PCR (see below).

Total RNA isolation and RT-PCR

The Trizol-LS procedure (Life Technologies) was used for total RNA isolations. Briefly, 0.5-2 μg of total RNA (based upon $OD_{260\ nm}$) was isolated from 10^6 non-sorted or sorted sheep peripheral blood cells, and 0.25-1 μg of total RNA was reverse transcribed using the first strand cDNA preamplification system (Life Technologies) and oligo dT. Volumes of 2.5 μL (11.9% of total cDNA) cDNA and 45 μL Platinum PCR Supermix (Life Technologies) were used in PCR reactions containing [0.2 μM]$_f$ of the PrP intron-spanning primers, shlh1a (5'-TAGTAACGGTC-CTCATAGTCATTG-3') and shexon2 (5'-CAACCAAGCT-GAAGCATCTGTCT-3') or the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) intron-spanning primers, GAPDH 171 (5'-GAGATGATGACCCTTTTGGC-3') and GAPDH 172 (5'-GTGAAGGTCGGAGTCAACG-3'). PCR amplification using either a Perkin Elmer 2400 or 9700 consisted of 5 minutes at 95°C, 30 cycles of the following: 15'' at 94°C, 30'' at 54°C, 30'' at 72°C, 7 minutes at 72°C, and 4°C indefinitely. A volume of 15 μL of the PCR reaction was mixed with 5 μL loading dye and analyzed on an ethidium bromide 1.5% TBE-agarose gel using an Alphaimaging System (Alpha-Innotech). Amplified PrP and GAPDH RT-PCR products were washed with centricon devices (Amicon) and sequenced at the University of Georgia

using 2 different PrP^c specific reverse primers: rev5a 5'-TCCACTGACTGTGGCTACCACCTT-3' or shlh1a (see above). Integrated densitometry values of PCR products were calculated using identical square areas.

Preparation of peripheral blood cells for evaluation of cell-surface expressed PrP

Leukocytes with platelets were isolated as described above except erythrocytes were added back for peripheral blood PrP cell-surface analysis. The following mAbs were added at 0.75 μg per 1×10^6 leukocytes (containing platelets and erythrocytes) per 50 μL FWB to separate tubes: 8H4 (PrP^c) IgG_1,[23] 5B2 (PrP^c) IgG_1,[24] 7A12 (PrP^c) IgG_1,[24] CAPP2A (platelets) IgG_1,[25] DH59B (polymorphonuclear cells and monocytes) IgG_1,[26] CAM36A (monocytes) IgG_1,[26] 17D1 ($CD4^+$ T-lymphocytes) IgG_1, anti-sheep RBC (Serotec) IgM, and 69A coliS (isotype mAb to 8H4) IgG_1. This was incubated 15-30 min on ice. Three washes with FWB were performed as previously stated. After the final centrifugation, 50 μL per 1×10^6 leukocytes of FITC-conjugated anti-mouse IgG1 antibody (Caltag) diluted 1:100 in FWB were added and incubated for 15-30 min on ice in the dark. Two washes were performed as stated above using SWB. After the final wash, the cells were resuspended into 1-2 mL 1× PBS/10mM EDTA pH 6.9 and analyzed immediately or the cells were resuspended into 1-2 mL 1× PBS with 2% formaldehyde and stored covered at 4°C until analysis could be performed. Analysis was performed on a FACSort flow cytometer equipped with a 488 nm argon laser (Becton-Dickinson). Peripheral blood cell populations were gated by size and fluorescence corresponding to the reactivity observed with the monoclonal antibodies CAPP2A, DH59B, CAM36A, 17D1, and anti-sheep RBC. Ten thousand events were acquired for each of the following individual cell populations: total PBMC, low forward scatter PBMC, high forward scatter PBMC, granulocytes, erythrocytes, and platelets.

Two-color flow cytometry analysis of cell-surface expressed PrP on specific PBMC phenotypes

PBMC were isolated by gradient purification as described above. The mAbs GC1A (CD4) IgG_{2a} [26], 7C2B (CD8) IgG_{2a}, GB54A (WC1) IgG_{2a} [26], Pig45A (sIgM) IgG_{2b}, BAQ44A (B2) IgG_{2b}, and 8H4 (PrP) IgG_1 were added at 0.75 μg per 1×10^6 PBMC per 50 μL and were incubated for 15-30 minutes on ice. Three washes with FWB were performed as stated above. Subsequently, 50 μL per 1×10^6 PBMC of the following secondary antibodies at the given dilutions in FWB were added to the corresponding wells: FITC-conjugated goat anti-mouse IgG_1 (Caltag) (1:100), PE-conjugated goat anti-mouse IgG_{2a} (Caltag) (1:250), PE-conjugated goat anti-mouse IgG_{2b} (Caltag) (1:100), PE-conjugated goat-anti mouse IgM (Southern Biochemical) (1:200), FITC-conjugated goat-anti-mouse IgG_{2b} (Caltag) (1:100), PE-conjugated goat anti-mouse IgG_1 (Caltag) (1:100), and 10 μL of FITC-conjugated My4 (CD14 specific from Coulter). Cells were incubated on ice in the dark for 15-30 min. Two washes with SWB were performed as stated above. Cells were resuspended in 200 μL 1× PBS/2% formaldehyde and analyzed on a FACSort flow cytometer. Ten thousands events were analyzed after gating for PBMC using side and forward scatter.

For statistical analysis of the two-color flow cytometry data, a Kolmogorov-Smirnov (KS) two-sample test was used for the two histograms resulting from gating the following populations: PBMC phenotype positive cells alone and PBMC phenotype positive cells that were also positive for PrP. KS statistics calculated by the CellQuest Software Program (Becton-Dickinson) on the whole histograms produced the index of similarity D/s(n) and a *p* value. The number of PrP positive cells was calculated by counting the combined PBMC phenotype positive and PrP positive cells in a defined quadrant minus the PBMC phenotype positive cells in the same defined quadrant. The percentage of PrP positive cells was calculated by dividing the number of PrP positive cells by the total number of cells analyzed (in this case 10,000) and multiplying by 100%. The mean and standard deviation of the percentage of PrP positive cells for each phenotype in normal and scrapie-infected sheep (n=5) were calculated, and statistical analysis was conducted using a Student's t-test (assuming unknown variances are not equal and a small sample size).

For proteinase K (PK) and phosphatidylinositol specific phospholipase C (PIPLC) treatments, 3×10^6 PBMC were pre-incubated with amounts of PK ranging from 5.0-50 μg for 15 min at room temperature in 1 mL FWB or with amounts of PIPLC ranging from 0.05-2.0 U for 1 hour at room temperature in 1mL FWB. Controls were kept at room temperature for the same time as the cells treated with PK and PIPLC were. The cells were washed four times with FWB with centrifugations at 1,000×g at room temperature. The cells were incubated with 2.25 μg of 8H4 or 69A ColiS (isotype control) per 3×10^6 PBMC and followed the same procedure above for PBMC antibody incubations and washings. Next, 50 μL of FITC-conjugated goat anti-mouse IgG_1 diluted 1:100 in FWB were added to the cells and incubated in the dark on ice for 15-30 min. Washings were performed as above with SWB. After the final wash, PBMC were resuspended with 1× PBS with 2% formaldehyde. Analysis was performed on a FACSort flow cytometer. PBMC were gated using side and forward scatter and 10,000 events were analyzed for each sample. Up to 100,000 events were analyzed on PBMC from scrapie-infected sheep.

Results

Flow cytometry sorting of peripheral leukocytes followed by RT-PCR

To identify the peripheral blood cell type containing PrP mRNA, leukocytes with platelets from a normal sheep were sorted based upon size into platelets, PBMC and basophils, and eosinophils/neutrophils using side and forward scatter on a FACsVantage flow cytometer.[27] Basophils can not be excluded from the PBMC/basophil

population based on size using side and forward scatter flow cytometry;[28] however, CBC differentials showed that this population contained 1-2% basophils. Purity analysis after sorting was 95.0%, 93.0%, 85.0% for platelets, PBMC/basophils, and eosinophils/neutrophils, respectively. Figure 1 shows RT-PCR results from 10^6 unsorted leukocytes, 10^6 sorted PBMC/basophils, 10^6 sorted eosinophils/neutrophils, and 2×10^6 sorted platelets. Reactions without the presence of reverse transcriptase (-RT) using GAPDH intron spanning primers (lanes 4, 8, 12, 16) or PrP intron-spanning primers (lanes 2, 6, 10, 14) were negative. RT-PCR products using GAPDH intron-spanning primers (354 bpr) were used as positive controls (lanes 3, 7, 11, and 15). The GAPDH RT-PCR product from leukocytes was sequenced and confirmed as GAPDH (Genbank accession #AF272837). RT-PCR products using PrP intron spanning primers (532bp) were detected in the non-sorted leukocytes (lane 1) and the PBMC/basophil sorted cells (lane 5) but not in the eosinophil/neutrophil or platelet sorted cells (lanes 9 and 13). The PrP RT-PCR product from leukocytes was sequenced and confirmed as PrP (Genbank accession #AF267507).

Since basophils could not be separated from PBMC by size or basophil-specific antibody, PBMC were density gradient purified to remove basophils. Figure 1 (lanes 17-20) shows the RT-PCR results from 10^6 gradient purified PBMC from a normal sheep. Reactions without the presence of reverse transcriptase (-RT) using GAPDH or PrP intron-spanning primers were negative (lanes 18 and 20). RT-PCR product using GAPDH intron-spanning primers served as a positive control (lane 19). The RT-PCR product using PrP intron spanning primers from purified PBMC is shown in lane 17. The signal intensity of the PrP RT-PCR product from 10^6 purified PBMC was similar to or slightly greater than the signal intensities of the PrP RT-PCR products in 10^6 leukocytes and 10^6 sorted PBMC/basophil cells. This demonstrates that the majority of PrP mRNA in leukocytes originates from PBMC.

Flow cytometry sorting of PBMC followed by RT-PCR

To identify the PBMC cell type containing PrP mRNA, normal sheep PBMC were gradient purified and sorted into T-lymphocytes and B-lymphocytes using monoclonal antibodies (see Design and Methods) and a FACS Vantage flow cytometer. PBMC were gated based on size using side and forward scatter, and this gated population was sorted based upon indirect-fluorescence labeling. Figure 2A shows flow cytometry results of PBMC incubated with T-lymphocyte and B-lymphocyte specific mAbs followed by FITC-conjugated secondary antibody (B-lymphocyte) and PE-conjugated secondary antibody (T-lymphocyte). Figures 2B, 2C, and 2D show the purity of sorted B-lymphocytes, T-lymphocytes, and unseparated PBMC, respectively. The purity of sorted cells from this representative normal sheep was 99.5%, 99.2%, and 99.6% for B-lymphocytes, T-lymphocytes, and unseparated PBMC, respectively. For five normal

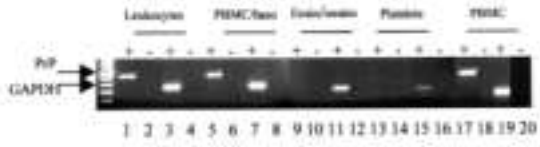


Figure 1. PrP mRNA is detected in PBMC using RT-PCR. An ethidium bromide 1.5% agarose gel showing the RT-PCR results of 10^6 non-sorted leukocytes (lanes 1-4),10^6 sorted mononuclear cells/basophils (lanes 5-8), 10^6 sorted eosinophils/neutrophils (lanes 9-12), $2x10^6$ sorted platelets (lanes 13-16), and 10^6 gradient purified PBMC (lanes 17-20). The plus(+) and minus(-) signs indicate RT-PCR reactions with or without reverse transcriptase (RT), and PrP and GAPDH arrows denote RT-PCR reactions using either PrP or GAPDH intron spanning primers. RT-PCR product sizes are 532 and 354 bp for PrP and GAPDH, respectively. A 100bpr DNA ladder is shown in the far left lane.

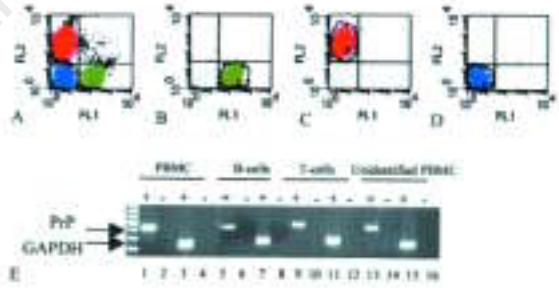
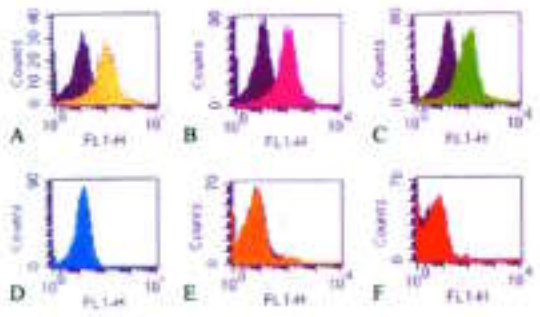


Figure 2 A-E. PrP mRNA is detected in sorted B-lymphocytes, T-lymphocytes, and unseparated PBMC using RT-PCR. Flow cytometry cell sorting of PBMC into B-lymphocytes, T-lymphocytes, and unseparated PBMC from a representative normal sheep. (A) Flow cytometry analysis of 10,000 PBMC cells after incubating with B-lymphocyte specific mAbs followed by FITC-labeled secondary antibodies (FL1, lower right quadrant) and T-lymphocyte specific mAbs followed by PE-labeled secondary antibody (FL2, upper left quadrant). The remaining unseparated PBMCs are shown in the lower left quandrant. (B) Flow cytometry purity analysis of 3,000 sorted B-lymphocytes was 99.5%(C) Flow cytometry purity analysis of 3,000 sorted T-lymphocytes was 99.2% (D) Flow cytometry purity analysis of 3,000 sorted unseparated PBMCs was 99.6%. (E) An ethidium bromide 1.5% agarose gel showing the RT-PCR results from the flow cytometry cell sorting in the upper panel of 10^6 non-sorted PBMCs (lanes 1-4), 10^6 sorted B-lymphocytes (lanes 5-8), 10^6 sorted T-lymphocytes (lanes 9-12), 10^6 sorted unseparated PBMCs (lanes 13-16). The plus (+) and minus (-) signs indicate RT-PCR reactions with or without reverse transcriptase (RT). PrP and GAPDH arrows denote RT-PCR reactions using PrP or GAPDH intron spanning primers. A 100bpr DNA ladder is shown in the far left lane.



Figures 3A-F. Flow cytometry analysis of cell-surface expressed PrP on peripheral blood cells from a normal sheep. Peripheral blood cells were either incubated with mAb 8H4 followed by incubation with FITC-conjugated secondary antibody or were incubated with the isotype control mAb 69A coliS followed by incubation with FITC-conjugated secondary antibody. Total PBMC, low forward scatter PBMC, high forward scatter PBMC, granulocytes, erythrocytes, and platelets were gated based on size and phenotype. (A) Histogram overlay of 10,000 total PBMC incubated with isotype mAb 69A coliS followed by FITC-conjugated secondary antibody (black) and 10,000 total PBMC incubated with 8H4 followed by FITC-conjugated secondary antibody (yellow). (B-F) same as (A), but analysis of (B) low forward scatter PBMC (fuchsia) (C) high forward scatter PBMC (green) (D) granulocytes (blue) (E) erythrocytes (orange), and (F) platelets (red).

sheep, the purity of sorted B-lymphocytes, T-lymphocytes, and unseparated PBMC was greater than 99.0%. RT-PCR results of 10^6 sorted T-lymphocytes, B-lymphocytes, unseparated PBMC, and non-sorted PBMC are shown in Figure 2E. Reactions without the presence of reverse transcriptase (-RT) using GAPDH (lanes 4, 8, 12, 16) or PrP intron-spanning primers (lanes 2,6,10,14) were negative. GAPDH RT-PCR products were detected in 10^6 unsorted PBMC (lane 3), 10^6 B-lymphocytes (lane 7), 10^6 T-lymphocytes (lane 11), and 10^6 unidentified PBMC (lane 15). PrP RT-PCR products were detected in 10^6 non-sorted PBMC (lane 1), 10^6 B-lymphocytes (lane 5), 10^6 T-lymphocytes (lane 9), and 10^6 unseparated PBMC (lane 13).

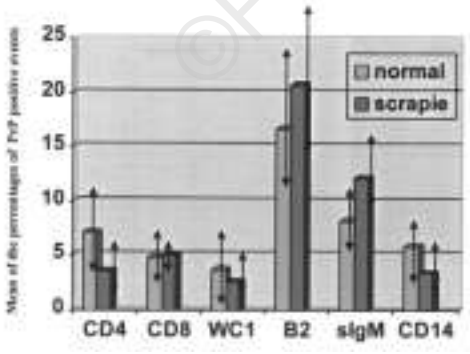


Figure 4. Bar graph representing the results from 2-color flow cytometry analysis of PrP cell-surface expression on specific PBMC phenotypes. The mean of the percentages of PrP positive cells (y-axis) is plotted versus specific PBMC positive phenotypes (x-axis) from five normal and five scrapie-infected sheep. The double-headed arrows represent the standard deviation of the mean of the percentages of PrP positive cells.

Flow cytometry analysis of cell-surface expressed PrP on peripheral blood cells

Cell-surface expression of PrP on peripheral blood cells was also investigated. A previously defined mAb (8H4), which reacts to ovine PrP[23] and binds to an epitope region on PrP encompassing amino acid residues 144-231,[24] was used to identify PrP expression on peripheral blood cells. Since the CBC differentials in these experiments had no detectable basophils, the gated population of PBMC/basophils based on size was considered to be only PBMC. Platelets, erythrocytes, PBMC and eosinophils/neutrophils were gated by size and phenotype (see *Design and Methods*). Total PBMC were further gated by size into high and low forward scatter PBMC. Figure 3 shows PrP cell-surface expression on PBMC from representative normal sheep (n=5). Figure 3A shows the histogram analysis of the total PBMC population after incubation with an isotype control mAb followed by the FITC-conjugated secondary antibody (black) and after incubation with 8H4 followed by FITC-conjugated secondary antibody (yellow). PBMC from normal sheep shifted approximately $\log_{10}$ in mean fluorescence (FL1) when incubated with the PrP mAb 8H4 followed by FITC-conjugated secondary antibody. In addition, PBMC gated for low and high forward scatter showed the same fluorescence shift as Figure 3A (see Figure 3B-C). However, neutrophils/eosinophils, erythrocytes, and platelets showed no fluorescence shifts with incubation of 8H4 followed by FITC-conjugated secondary antibody, and the resulting histograms overlapped the isotype control mAb (see Figure 3D-F).

Peripheral blood cells from 5 scrapie-infected sheep were also evaluated for cell-surface expressed PrP. Using the PrP mAb (8H4), the scrapie-infected sheep showed no significant differences when compared to PrP cell-surface expression of normal sheep (n=5) in which only PBMC harbored cell-surface expressed PrP and shifted $\log_{10}$ in mean fluorescence. In addition, mAb (5B2), which binds to PrP between 34-52 amino acid residues,[24] and mAb (7A12), which binds to PrP between 90-145 amino acid residues,[24] detected cell-surface PrP on PBMC and not on other peripheral blood cells in normal and scrapie-infected sheep.

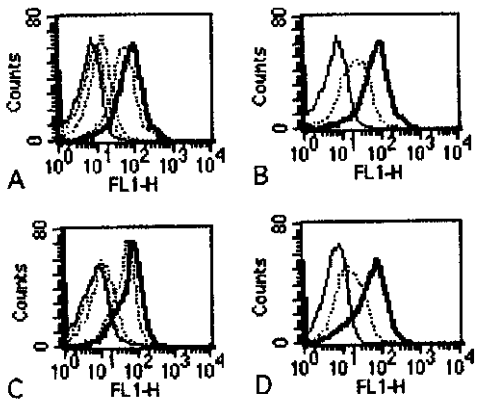
Two-color flow cytometry analysis of cell-surface expressed PrP on specific PBMC phenotypes

Since PrP was detected on the cell surface of PBMC using the mAb 8H4 and sheep cross-reacting PBMC mAbs exist, 2-color flow cytometry analysis was performed to examine possible differences in PrP cell-sur-

face expression on specific PBMC phenotypes. Uniform mean fluorescence shifts in cell-surface expression of PrP were observed for all the PBMC phenotypes evaluated in this study. Furthermore, Kolmogorov-Smirnov (KS) statistics[29] revealed the resulting histograms representing PrP positive and the specific PBMC phenotype positive cells were significantly different from the specific PBMC phenotype positive cells alone (p <0.001) in all cases. The mean and standard deviation of the percentages of PrP positive cells were calculated (see Design and Methods) for each PBMC phenotype from five normal and five scrapie-infected sheep and plotted in Figure 4. Regardless of disease status, B2 positive cells showed a highly significant difference (p <0.05) in the mean percentage of PrP positive cells versus CD4, CD8, WC1, and CD14 positive cells. Also, sIgM positive events in scrapie-infected sheep showed a highly significant difference (p<0.05) in the mean percentage of PrP positive cells versus CD8, WC1, and CD14 positive cells in normal and scrapie-infected sheep.

Flow cytometry analysis of cell-surface expressed PrP after proteinase K (PK) or phosphatidyl-inositol phospholipase C (PIPLC) treatment of PBMC from normal and scrapie-infected sheep

Since PrP was found on PBMC of normal and scrapie-infected sheep and cell-surface PrP^c is PK and PIPLC sensitive,[30,31] PBMC from normal and scrapie-infected sheep were treated with PK or PIPLC and evaluated for cell-surface PrP^c using mAb 8H4 and flow cytometry (Figure 5). Increasing amounts of PK of 5, 15, and 50 μ/mL (Figure 5A and 5C, dotted line histograms from right to left) on PBMC from a representative normal (5A) and scrapie-infected sheep (5C) exhibited a dose response using 8H4 and FITC-conjugated secondary antibody. The histogram representing 50 μg PK overlapped with the histograms representing FITC-conjugated secondary antibody alone (see Figure 5A and 5C) and the isotype control (data not shown). Results from five different normal and scrapie-infected sheep were identical to results shown in Figures 5A and 5C. Histogram analysis of cell-surface expressed PrP^c on PBMC from normal and scrapie-infected sheep after PIPLC treatment at 0.5 U/mL per 3×10^6 PBMCs showed approximately half a $\log_{10}$ fluorescence shift (FL1) when compared to PBMC which had not been treated with PIPLC (Figures 5B and 5D). PIPLC titrations (0.05-2U) on equivalent numbers of PBMCs or incubated for a longer time revealed no differences in mean fluorescence shift indicating there was no PIPLC concentration dependence using these amounts of PIPLC under these conditions (data not shown).



Figures 5A-D. Flow cytometry analysis of PBMC-surface expressed PrP following proteinase K (PK) and phosphatidylinositol specific phospholipase C (PIPLC) treatments from normal and scrapie-infected sheep. (A) Histogram overlay showing PBMC isolated from a normal sheep and treated without PK (thick solid line filled histogram) and with increasing amounts of PK 5, 15, and 50μg/mL (dotted line histograms from right to left) followed by incubation with 8H4 and FITC-conjugated secondary antibody. The FITC-conjugated secondary antibody alone without PK treatment is shown by the thin solid line histogram. (B) Histogram overlay showing PBMC isolated from a normal sheep and treated without PIPLC (solid line filled histogram) and with PIPLC (dotted line histogram) followed by incubation with 8H4 and FITC-conjugated secondary antibody. The FITC-conjugated secondary antibody alone without PIPLC treatment is shown by the thin solid line histogram. (C) Same as A except PBMC were isolated from a scrapie-infected sheep. (D) Same as B except PBMC were isolated from a scrapie-infected sheep.

Discussion

PrP mRNA was detected in PBMC but not in granulocytes or platelets of normal sheep. Consistent with PBMC harboring PrP mRNA, cell-surface PrP^c was detected on peripheral blood mononuclear cells of normal sheep but was not detected in granulocytes, erythrocytes or platelets. These observations are consistent with the observation that PrP^c was detected in human PBMC and detected at much lower levels in erythrocytes and granulocytes.[9,12] However, in contrast to the situation with resting sheep platelets, PrP^c mRNA was detected in resting human platelets,[11] and cell-surface PrP^c was detected in resting and activated human platelets.[12] Since PrP^c was found to be released from resting human platelets,[11] it is possible that resting sheep platelets release more PrP^c from their cell-surface. However, since PrP mRNA was not detected in resting sheep platelets using RT-PCR and intron spanning primers, differences in PrPc cell-surface expression between human and sheep platelets could be related to inherent species differences.

Regardless of scrapie-status, PrP^c was detected on PBMC and not other peripheral blood cells. In addition, PrP^c was detected on all PBMC phenotypes evaluated in this study using two-color flow cytometry analysis. There were significant differences in the mean of the percentage of PrP^c positive cells when comparing 1) B2 positive cells versus CD4, CD8, WC1, and CD14 positive cells regardless of disease (n=5) and 2) sIgM positive

cells in scrapie-infected sheep versus CD8, WC1, and CD14 positive cells in normal and scrapie-infected sheep (n=5). These results suggest that regardless of disease, B-lymphocytes positive for the PBMC phenotype B2 show an increase in PrP^c cell-surface expression over CD4, CD8, WC1 positive T-lymphocytes and CD14 positive monocytes. And, B-lymphocytes positive for the PBMC phenotype sIgM in scrapie-infected sheep show an increase in PrP^c cell-surface expression over CD8, WC1 positive T-lymphocytes and CD14 positive monocytes from normal and scrapie-infected sheep. However, in regard to disease, no significant differences were observed in PrP^c cell-surface expression when comparing normal and scrapie-infected sheep in a specific PBMC phenotype.

Although the peripheral lymph nodes of scrapie-infected sheep contain a high infectious titer[14] and PrP^{Sc},[15,16,32-35] infectious titers have not been detected in the peripheral blood of scrapie-infected sheep.[14] Under our conditions, cell-surface expressed PrP on PBMC was PK-sensitive and PIPLC-sensitive regardless of the scrapie-status of the sheep. There are several possibilities to account for the lack of PK-resistant PrP (or PrP-res) or PIPLC-resistant PrP on PBMC from scrapie-infected sheep. First, PrP-res may accumulate in the follicular dendritic cells[36,37] and not be transferred to the cell-surface of circulating PBMC at detectable levels. This may be a distinct possibility since PrP-res can be detected using Western blot analysis from 3×10^6 lymph node dissociated cells from a scrapie-infected sheep, whereas PrP-res cannot be detected from 3×10^6 PBMC from a scrapie-infected sheep (unpublished data). Second, although the mAb 8H4 reacts to PrP-res on Western blots (unpublished data), 8H4 may not recognize native PrP-res in flow cytometry analysis. Evaluation of this possibility will proceed when native state PrP-res specific mAbs are produced. Third, although flow cytometry is extremely sensitive (1,500 molecules/cell[27] or the attomole level), other methods such as capillary electrophoresis resolved competitive immunoassay,[13] time resolved fluoroimmunoassay assay,[38] and bioassays in conventional and transgenic mice[4,39] may aid in the enhanced detection of PrP-res in cell lysates.

These results show that regardless of the scrapie-status of the sheep, PrP^c is transcribed in resting PBMC, and is expressed on the cell-surface of resting PBMC. In addition, PrP^c was not detected on the cell surface of erythrocytes, granulocytes, or platelets which suggests that PrP^c does not transfer from the cell surface of PBMC to the cell surface of other peripheral blood cells through cell to cell contact. And, although PK-resistant or PIPLC-resistant PrP was not detected on PBMC of scrapie-infected sheep, PK-sensitive and PIPLC-sensitive PrP was detected on PBMC of normal and scrapie-infected sheep. Because of the abundance of PrP^c on PBMC, future diagnostic tests using PK and PIPLC to discriminate between protease sensitive and resistant PrP must be carefully evaluated.

Contributions and Acknowledgments

LMH and KO'R contributed equally to this work from conception to submitted manuscript, and they should be considered as the principal authors. The remaining authors qualified for authorship according to the WAME criteria, and have taken specific responsibility for the following parts of the content: WCD contributed to acquisition of data and analysis of data; JW collected clinical data; MSS and PG helped with data acquisition; DPK contributed to the conception and experimental design. Order of authorship. Authors are listed according to a criterion of decreasing individual contribution to the work, with the following exceptions: the last author had a major role in interpreting the data and preparing the article.

I thank Emma Karel, Linda Hamburg, Mary Jo Hamilton, Robert Finch, Duane Chandler, and Pete Steiner for their technical and animal handling expertise. I also thank Tim Baszler, Hong Li, and Phil Cheevers for their critical reading of this manuscript. Finally, I thank Travis McGuire, Wenbin Tuo and Curtis Hoesing for discussing various aspects of this work.

Funding

This work was supported by USDA/ARS CWU# 5348-32000-015-00D.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Prof. Adriano Aguzzi, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Prof. Aguzzi and the Editors. Manuscript received October 17, 2000; accepted December 28, 2000.

Potential implications for clinical practice

Because of the abundance of cellular prion protein[40-42] on peripheral blood mononuclear cells, future diagnostic tests for scrapie and/or other prion diseases using proteinase K (PK) and phosphatidylinositol phospholipase C (PIPLC) must be carefully evaluated.

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