Expression of PrP\textsuperscript{C} on cellular components of sheep blood

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PrP\textsuperscript{C}, a glycosylphosphatidylinositol-linked glycoprotein, plays a central role in the pathogenesis of transmissible spongiform encephalopathies (TSEs), undergoing a conformational alteration to the disease-associated isoform, commonly designated PrP\textsuperscript{Sc}. PrP\textsuperscript{C} is expressed in many tissues other than the nervous system, although its precise function(s) remains unclear. It has previously been demonstrated that TSEs can be transmitted by blood transfusion in sheep. The aim of this work was to identify which components of blood carried the infection. As an initial step, the distribution of PrP\textsuperscript{C} on cellular components of sheep blood was examined to identify potential targets for infection. Cell-surface expression of PrP\textsuperscript{C} was found only on peripheral blood mononuclear cells (PBMCs); however, platelets also contained significant amounts of intracellular PrP\textsuperscript{C}. The level of PrP\textsuperscript{C} expressed on the cell surface of PBMCs was influenced by PrP genotype, with the highest levels found in scrapie-susceptible VRQ/VRQ sheep and the lowest levels in scrapie-resistant ARR/ARR sheep. In susceptible sheep, PrP\textsuperscript{C} was expressed at varying levels on all major subsets of PBMCs, with the highest levels on the CD21\textsuperscript{+} subset of B cells, and PrP expression was upregulated dramatically on CD21\textsuperscript{+} B cells in some scrapie-infected sheep.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) or prion diseases are neurodegenerative diseases that occur in a variety of species, including sheep (scrapie), cattle (bovine spongiform encephalopathy, BSE) and humans (e.g. Creutzfeld–Jakob disease, CJD). A characteristic feature of these diseases is the accumulation of PrP\textsuperscript{Sc}, a post-translationally modified form of the host glycoprotein PrP\textsuperscript{C}, in the central nervous system (CNS). As PrP\textsuperscript{Sc} and infectivity co-purify (Bolton et al., 1982), the presence of PrP\textsuperscript{Sc} is considered a marker for TSE infection. PrP\textsuperscript{C} is expressed in many different tissues, with the highest levels found in neurons of the CNS (Prusiner, 1998), but its function is still unclear. PrP-null mice are resistant to TSE infection, emphasizing the key role of PrP in the pathogenesis of these diseases.

Replication of TSE agents and deposition of PrP\textsuperscript{Sc} in lymphoreticular tissues precedes infection of the CNS in certain TSE diseases, e.g. scrapie, variant CJD (vCJD) and many experimental rodent TSE models (Eklund et al., 1967; Hadlow et al., 1982; Hill et al., 1999). In lymphoid tissues, PrP\textsuperscript{Sc} deposits are localized to germinal centres, and follicular dendritic cells have been shown to play a key role in replication of infectivity (Bruce et al., 2000). Infection of lymphoid tissues raises the possibility of haematogenous spread of infectivity, as lymphocytes recirculate between blood and secondary lymphoid organs. Although this route is not believed to be significant for neuroinvasion (Blättler et al., 1997), it probably explains the widespread dissemination of infection in lymphoid tissues seen, for example, in clinical cases of scrapie. Until recently, it has proved difficult to show conclusively that blood from natural TSE cases (human or animal) contains infectivity or PrP\textsuperscript{Sc}, despite the fact that low levels of infectivity have been demonstrated in the blood of experimental rodent TSE models (Brown, 1995). However, it has now been shown that both natural scrapie and experimental BSE can be transmitted between sheep by transfusion of whole blood or buffy coat (Houston et al., 2000; Hunter et al., 2002). In addition, there has been a report of a vCJD case that may have resulted from an infected blood transfusion (Llewelyn et al., 2004) and a more recent report of a pre-clinical vCJD case detected in a patient heterozygous for codon 129 of the prion-protein gene after blood transfusion (Peden et al., 2004).

The distribution of infectivity in the blood of scrapie-infected sheep is not known, but experiments in rodent models have shown that the highest levels are found in buffy coats (which contains leukocytes and platelets), followed by plasma (Brown et al., 1998, 1999). In hamster scrapie, very little infectivity is found in association with purified platelets (Holada et al., 2002). Attempts have been made to detect PrP\textsuperscript{Sc} in blood by methods such as immunocapillary electrophoresis (Schmerr et al., 1999), immunocytochemistry (Herrmann et al., 2002) and Western blotting...
(Wadsworth et al., 2001), but the results so far have been negative or inconclusive. The distribution of PrP<sup>C</sup> in different blood components may help to define potential targets for infection, although cautious interpretation of the results is required, because there is not a precise correlation between infection of tissues and their expression of PrP<sup>C</sup>. Interestingly, comparative studies of healthy animals of different species (mouse, hamster, human, sheep and cattle) have revealed marked differences in the distribution and expression levels of PrP<sup>C</sup> on blood cells (Barclay et al., 2002; Holada & Vostal, 2000). In sheep, cell-surface expression of PrP<sup>C</sup> was confined to peripheral blood mononuclear cells (PBMCs) and the distribution of PrP mRNA followed the same pattern (Herrmann et al., 2001). In humans, the highest levels of PrP<sup>C</sup> expression were found on platelets and PBMCs, and much lower levels were demonstrated on red cells and neutrophils (Barclay et al., 1999, 2002). Human platelets also appear to contain PrP<sup>C</sup> in intracellular granules and may be responsible for release of soluble PrP<sup>C</sup> into plasma (Perini et al., 1996). The function of PrP<sup>C</sup> in blood-associated cells is unknown, although experiments with PBMCs from healthy and PrP-null mice have suggested that it may play a role in lymphocyte activation and proliferation (Cashman et al., 1990; Mabbott et al., 1997).

In the absence of specific methods to distinguish PrP<sup>C</sup> from PrP<sup>C</sup> in blood, changes in the distribution or expression levels of PrP on blood cells during scrapie infection may provide indirect evidence of cell types transporting, or infected with, the scrapie agent. Here, we have described experiments that have characterized in greater detail the expression of PrP in sheep-blood components, including subsets of PBMCs, from uninfected and scrapie-infected sheep. In uninfected sheep, we showed that platelets contained significant amounts of intracellular PrP<sup>C</sup> and that the level of PrP<sup>C</sup> expressed on PBMCs differed between scrapie-susceptible and -resistant sheep. We also demonstrated for the first time that PrP<sup>C</sup> expression appears to be greatly upregulated on the CD21<sup>+</sup> subset of B cells in some scrapie-infected sheep.

**METHODS**

**Sheep.** The sheep used in these experiments originated from the Department for Environment, Food and Rural Affairs (UK) scrapie-free flock, which was established in 1998 from sheep imported from New Zealand and has been maintained in strict isolation from UK livestock. Male and female Cheviot and Poll Dorset sheep between 1 and 3 years old were used. PrP<sup>C</sup>-gene polymorphisms at codons 136 (A/V), 154 (R/H) and 171 (R/Q) were confirmed for each animal by automated DNA sequencing of the PCR-amplified coding region. In one experiment, groups of VQO/VQO and VQO/ARQ Poll Dorset sheep were infected experimentally with scrapie by subcutaneous inoculation of 2 ml 10% SSBP/1 brain homogenate, as described previously (Houston et al., 2002). These sheep were housed throughout the experiment until clinical signs of scrapie developed and were then euthanized in accordance with UK Home Office guidelines.

**Isolation of different cellular components from sheep blood.** Blood samples were obtained by jugular venipuncture, using 3-8% (w/v) sodium citrate solution (Sigma) as anticoagulant. Cellular components of sheep blood were separated by density-gradient centrifugation on Histopaque-1083 (1-083 g ml<sup>–1</sup>; Sigma) or Polymorphprep (Nycomed), with slight variations in the methods depending on the application.

Polymorphprep, a 1-11 g ml<sup>–1</sup> gradient containing sodium diatrizoate (13-8%) and dextran 500 (8-0%), is designed for use on human blood and separates polymorphonuclear cells (granulocytes) from PBMCs, with each cell type appearing as a distinct band at different levels of the gradient. In brief, 25 ml blood was layered undiluted on to Polymorphprep gradients and centrifuged at 500 g for 25 min, according to the manufacturer’s instructions. All steps were performed at ambient temperature unless otherwise stated. PBMCs were harvested from the upper band and granulocytes from the lower band and diluted in PBS (pH 7-4) containing 9 mM EDTA (PBS/EDTA). Pelleted erythrocytes were also collected and diluted with PBS/EDTA. To remove residual PBMCs, the granulocytes were layered over Histopaque-1083, centrifuged at 1000 g for 25 min and the pelleted cells were washed twice by centrifugation at 300 g for 15 min, first in PBS/EDTA and then in PBS alone. PBMCs were washed twice as described for the granulocytes. After the first wash, the supernatant was collected and centrifuged at 7800 g for 5 min to isolate platelets. Platelets were then washed twice in PBS/EDTA at 7800 g for 5 min.

For some experiments, PBMCs, platelets and granulocytes were isolated by using Histopaque-1083. Briefly, blood was diluted 1:1 with PBS, layered over Histopaque-1083 and centrifuged at 3000 g for 30 min. PBMCs at the gradient interface were collected and washed three times by centrifugation in PBS, as above. To isolate platelets for permeabilization, harvested PBMCs were washed in platelet buffer [PBS containing 9 mM EDTA, 10 ng prostacyclin ml<sup>–1</sup> (Sigma), 1% (w/v) BSA and 0-1% (w/v) sodium azide] for 10 min at 400 g. The supernatant from this wash was collected and washed twice in platelet buffer at 1000 g for 10 min. Granulocytes that passed through the Histopaque gradient were isolated by lysing the erythrocytes with ammonium chloride lysis buffer [155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0-1 mM EDTA (pH 7-2)]. Cells were incubated in the buffer for 10 min at ambient temperature and then centrifuged at 300 g for 10 min at 4°C, followed by two washes at 300 g for 5 min at 4°C, first in PBS/EDTA, then in PBS alone.

**Preparation of erythrocyte membranes (‘ghosts’).** Erythrocyte-membrane ‘ghosts’ were prepared by lysing the cells pelleted from the Polymorphprep gradient in 3 mM sodium dihydrogen orthophosphate (pH 8-0) containing 1 mM dithiothreitol. Briefly, 50 ml ice-cold lysis buffer was added to 1 ml packed erythrocytes on ice, vortexed and left on ice for 5 min. Cells were centrifuged at 300 g for 10 min at 4°C to remove non-lysed cells and contaminating leukocytes. Membranes were pelleted by centrifuging the supernatant at 20000 g for 40 min at 4°C and washed twice with cold lysis buffer and once with cold PBS.

**Monoclonal antibodies (mAbs).** The mAbs used for identification of PrP<sup>C</sup> and for phenotyping subsets of PBMCs, along with their isotypes and epitope specificity, are listed in Table 1. FH11 (TSE Resource Centre, IAH, Compton, UK) was raised against full-length, *Escherichia coli*-expressed, recombinant bovine PrP, 4F2 was a gift from Andreas Stuke (Deutsches Primatenzentrum, Göttingen, Germany) and 8G8 and 6H4 were purchased from Spi-Bio and Prionics, respectively. Dr C. J. Howard and the Monoclonal Antibody Production Section, IAH, Compton, UK, supplied CC21, CC32, CC125, CC-G33, II-A51 and 86D. TD14 came from Professor J. Hopkins, University of Edinburgh, UK, and Du2-104 from Dr W. Hein, Basel Institute for Immunology, Switzerland. Antibodies 44-97 and 25-69 were supplied by Dr E. Meeusen, University of Melbourne, Australia, and CAPP2A was purchased from VMRD.
PBMCs, granulocytes, erythrocytes and platelets were analysed by flow cytometry using one-colour indirect immunofluorescence. PBMCs, granulocytes and erythrocytes were distributed at 10^6 cells per well and platelets at 10^7 per well in a 96-well microtitre plate. Each fraction was incubated with the primary antibodies 4F2 (1 μg ml^{-1}), TD14 (1:10 dilution of culture supernatant) and CAPP2A (5 μg ml^{-1}) for 10 min at room temperature. All antibodies used for flow cytometry were diluted in PBS containing 1 % (w/v) BSA and 0-1 % (w/v) sodium azide, except for the platelet studies, where the antibodies were diluted in platelet buffer. PBMCs, granulocytes and erythrocytes were washed three times in PBS containing 1 % (w/v) BSA and 0-1 % (w/v) sodium azide at 400 g for 2 min. Platelets were washed three times in platelet buffer at 1000 g for 2 min. Cells were incubated for 10 min at room temperature with secondary goat anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate (FITC), diluted 1:200 (Southern Biotechnology Associates) and then washed twice, as above. Negative controls included samples in which primary and/or secondary antibodies had been omitted and samples in which the primary antibody was replaced with an isotype-matched antibody of irrelevant specificity.

In some experiments, platelets and PBMCs were permeabilized to demonstrate intracellular PrP^C. Briefly, 100 μl Perm-2 solution (diluted 1:10 in distilled water; Becton Dickinson) was added to the cells and incubated for 10 min at room temperature and the cells were washed twice as described above. Cells were then labelled for PrP^C, as above. To demonstrate that cells were permeabilized adequately, Alexa fluor 568 phalloidin (Molecular Probes Europe BV) at a dilution of 1:40 was used as a positive control to stain the actin cytoskeleton.

For two-colour indirect-immunofluorescence analysis of PrP^C expression on subsets of PBMCs, samples were incubated for 10 min at room temperature with 8G8 (1 μg ml^{-1}) and one of the following subset-specific markers: 44-97, IL-A51, 86D, CC-G33, 25-69, CC125, CC32, CC21 or DU2-104 (Table 1). Antibodies were supplied as hybridoma-culture supernatants and were diluted 1:10 as above, apart from Du2-104, which was used undiluted. After washing as above, samples were incubated with isotype-specific secondary antibodies (Southern Biotechnology Associates): either FITC-conjugated goat anti-mouse IgG1 (diluted 1:100) or FITC-conjugated goat anti-mouse IgM (diluted 1:100), together with phycoerythrin-conjugated goat anti-mouse IgG2a (diluted 1:200) for 10 min at room temperature, then washed as above.

Cells were resuspended in 100 μl 1 % (w/v) paraformaldehyde in PBS and stored overnight at 4 °C before analysis. As described previously (Barclay et al., 2002), samples were analysed on a flow cytometer (FACSCalibur; Becton Dickinson) equipped with a previously calibrated 488 nm argon laser. Software used for analysis was WinMDI 2.8 (The Scripps Research Institute, CA, USA). For each sample studied, at least 10 000 cells of interest were acquired by appropriate gating based on light-scattering characteristics and/or expression of cell-specific markers.

Mean fluorescence intensity (MFI) was calculated by the WinMDI software as either the geometric mean of total PrP fluorescence for one-colour staining or the geometric mean of the PrP fluorescence in the gated cell subset for two-colour staining. The MFI for negative controls (PrP-specific mAbs omitted) was calculated for comparison. The negative-control values were subtracted from the mean PrP fluorescence to give a value corresponding to PrP-specific fluorescence.

Table 1. mAbs used for flow cytometry and Western blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Subclass</th>
<th>Specificity</th>
<th>Epitope (aa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH11</td>
<td>IgG2b</td>
<td>PrP</td>
<td>54–57</td>
<td>C. R. Birkett &amp; J. Langeveld (personal communication)</td>
</tr>
<tr>
<td>4F2</td>
<td>IgG2b</td>
<td>PrP</td>
<td>61–76</td>
<td>Krasemann et al. (1999)</td>
</tr>
<tr>
<td>8G8</td>
<td>IgG2a</td>
<td>PrP</td>
<td>95–110</td>
<td>Krasemann et al. (1999)</td>
</tr>
<tr>
<td>6H4</td>
<td>IgG1</td>
<td>PrP</td>
<td>144–152</td>
<td>Korth et al. (1997)</td>
</tr>
<tr>
<td>44-97</td>
<td>IgG1</td>
<td>CD4 (T-cell subset)</td>
<td>–</td>
<td>Maddox et al. (1985)</td>
</tr>
<tr>
<td>IL-A51</td>
<td>IgG1</td>
<td>CD8 (T-cell subset)</td>
<td>–</td>
<td>MacHugh &amp; Sopp (1991)</td>
</tr>
<tr>
<td>86D</td>
<td>IgG1</td>
<td>γδ T-cell receptor</td>
<td>–</td>
<td>Mackay et al. (1989)</td>
</tr>
<tr>
<td>CC-G33</td>
<td>IgG1</td>
<td>CD14 (monocytes)</td>
<td>–</td>
<td>Sopp et al. (1996)</td>
</tr>
<tr>
<td>25-69</td>
<td>IgG1</td>
<td>slgM (B cells)</td>
<td>–</td>
<td>Maddox et al. (1987)</td>
</tr>
<tr>
<td>CC125</td>
<td>IgG1</td>
<td>CD11b (B-cell subset)</td>
<td>–</td>
<td>Hall et al. (1993)</td>
</tr>
<tr>
<td>CC32</td>
<td>IgG1</td>
<td>CD62 (lymphocyte subsets)</td>
<td>–</td>
<td>Howard et al. (1992)</td>
</tr>
<tr>
<td>CC21</td>
<td>IgG1</td>
<td>CD21 (B cells)</td>
<td>–</td>
<td>Sopp (1996)</td>
</tr>
<tr>
<td>DU2-104</td>
<td>IgM</td>
<td>CD72 (B cells)</td>
<td>–</td>
<td>Young et al. (1997)</td>
</tr>
<tr>
<td>CAPP2A</td>
<td>IgG1</td>
<td>CD41/CD61 (platelets)</td>
<td>–</td>
<td>Mateo et al. (1996)</td>
</tr>
<tr>
<td>TD14</td>
<td>IgG1</td>
<td>CD45 (leukocytes)</td>
<td>–</td>
<td>Bembridge et al. (1993)</td>
</tr>
</tbody>
</table>

Analysis of PrP^C expression on blood-cell populations by flow cytometry. PBMCs, granulocytes, erythrocytes and platelets were analysed by flow cytometry using one-colour indirect immunofluorescence. PBMCs, granulocytes and erythrocytes were distributed at 10^6 cells per well and platelets at 10^7 per well in a 96-well microtitre plate. Each fraction was incubated with the primary antibodies 4F2 (1 μg ml^{-1}), TD14 (1:10 dilution of culture supernatant) and CAPP2A (5 μg ml^{-1}) for 10 min at room temperature. All antibodies used for flow cytometry were diluted in PBS containing 1 % (w/v) BSA and 0-1 % (w/v) sodium azide, except for the platelet studies, where the antibodies were diluted in platelet buffer. PBMCs, granulocytes and erythrocytes were washed three times in PBS containing 1 % (w/v) BSA and 0-1 % (w/v) sodium azide at 400 g for 2 min. Platelets were washed three times in platelet buffer at 1000 g for 2 min. Cells were incubated for 10 min at room temperature with secondary goat anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate (FITC), diluted 1:200 (Southern Biotechnology Associates) and then washed twice, as above. Negative controls included samples in which primary and/or secondary antibodies had been omitted and samples in which the primary antibody was replaced with an isotype-matched antibody of irrelevant specificity.

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Mean fluorescence intensity (MFI) was calculated by the WinMDI software as either the geometric mean of total PrP fluorescence for one-colour staining or the geometric mean of the PrP fluorescence in the gated cell subset for two-colour staining. The MFI for negative controls (PrP-specific mAbs omitted) was calculated for comparison. The negative-control values were subtracted from the mean PrP fluorescence to give a value corresponding to PrP-specific fluorescence.

Analysis of PrP^C expression in blood-cell populations by Western blotting. Fractions were prepared by using Histopaque-1083, as described above. All pellets were stored at −80 °C until required. Samples were thawed and diluted to an appropriate volume with PBS. An equal volume of 2 × NuPAGE lithium dodecyl sulphate sample buffer (Invitrogen) was added to the sample, mixed and a 1:10 final dilution of NuPAGE sample reducing agent (Invitrogen) was added and mixed. All samples were heated for 5 min at 100 °C on a heating block before electrophoresis on 10 % Bis/Tris NuPAGE Novex gels (Invitrogen) using standard protocols. NuPAGE antioxidant (Invitrogen) was added to the running buffer at a 1:400 dilution. Gels were electroblotted onto an Immobilon-P transfer membrane (Millipore) and blocked for 1 h in 5 % (w/v) milk powder in PBS containing 0-05 % (v/v) Tween 20 (PBST). PrP was detected by probing the membrane with 4F2 [1 μg ml^{-1} in PBST containing 0-5 % (w/v) milk powder] overnight at 4 °C. The membrane was then washed with PBST for 30 min (with six changes of buffer) and subsequently incubated for 1 h with goat anti-mouse IgG conjugated to horseradish peroxidase [diluted 1:7500 in PBST containing 0-5 % (w/v) milk powder]. Blots were
washed as before and developed by using Supersignal West Pico chemiluminescent substrate (Perbio Science UK) and visualized on Hyperfilm ECL (Amersham Biosciences).

RESULTS

Expression of PrP<sup>C</sup> in cellular components of blood

Expression of PrP<sup>C</sup> on the surface of sheep granulocytes, PBMCs, platelets and erythrocytes was analysed by flow cytometry using a variety of PrP-specific mAbs (FH11, 8G8, 6H4 and 4F2) that bound to different epitopes on PrP (see Table 1). All cells were gated according to their light-scattering characteristics (data not shown).

The efficiency of the separation of different fractions was confirmed by staining with antibodies TD14 (CD45) and CAPP2A (CD41/CD61). CD45 was expressed on 100% of leukocytes (granulocytes and PBMCs), but not on platelets or erythrocytes (red blood cells) (Fig. 1a).

Fig. 1. Cellular distribution of PrP<sup>C</sup> in uninfected sheep blood. (a) Cell-surface PrP<sup>C</sup> expression on PBMCs, granulocytes (GRAN), platelets (PLT) and red blood cells (RBC) analysed by flow cytometry. Cells were stained by using antibodies to PrP (4F2, black line), CD45 (TD14, dashed line) and CD41/CD61 (CAPP2A, grey line). Filled area, negative controls (no antibodies). (b) Western blot analysis of PrP<sup>C</sup> expression in sheep-blood cells, probed with mAb 4F2. (i) Lane 1, normal sheep-brain homogenate (equivalent to 80 ng tissue); lane 2, PBMCs (~1.6 × 10<sup>6</sup> cells); lanes 3 and 4, doubling dilutions of PBMCs; lane 5, platelets (~3.2 × 10<sup>8</sup> cells); lanes 6 and 7, doubling dilutions of platelets. Exposure time, 5 min. (ii) Red blood-cell membranes (equivalent to ~1.6 × 10<sup>8</sup> cells). Exposure time, 4 h. (c) Flow-cytometry analysis of intracellular PrP<sup>C</sup> in platelets and PBMCs. Cells were stained with 4F2 (thick line) with or without permeabilization. Filled area, isotype-matched negative controls.
CD41/CD61 was expressed on >95% of ovine platelets. The erythrocyte population was negative for CD41/CD61, but a small percentage of leukocytes was positive. This has been observed previously (Mateo et al., 1996; Sopp & Howard, 1997) and may be due to adherence of activated platelets or platelet fragments to the cells. No erythrocyte-specific markers were available; however, as the isolated population was negative for CD45 and CD41/CD61, it was assumed to be pure. Polymorph prep did not give a complete separation of granulocytes and PBMCs, presumably because sheep leukocytes have slightly different densities from the human equivalents. However, these two populations could also be distinguished readily by their light-scattering characteristics during flow cytometry.

Regardless of the mAb used, significant amounts of PrP C were found only on PBMCs and not on the other cell populations (Fig. 1a). These results have been reported previously (Barclay et al., 2002) and are in agreement with the findings of other investigators (Herrmann et al., 2001). However, when the same cell populations were analysed by Western blotting, significant levels of PrP C were detected in platelets (Fig. 1b), suggesting that it is in a predominantly intracellular location in these cells. The three bands representing diglycosylated, monoglycosylated and unglycosylated PrP C were clearly visible at the highest concentration of PBMCs (as for brain-derived PrP C). In both platelets and PBMCs, the diglycosylated form of PrP C was predominant, but in platelets, there appeared to be much less of the mono- and unglycosylated forms. In addition, migration of the monoglycosylated protein appeared to be slightly faster in platelets than in PBMCs. This suggested that there are cell type-specific differences in the glycosylation pattern of PrP C. A band with a molecular mass equivalent to that of diglycosylated PrP C was also detected on erythrocyte membranes after prolonged exposure times [Fig. 1b(ii)], suggesting that this cell type may also express low levels of PrP C. No PrP C was detected in sheep granulocytes (data not shown), in line with the flow-cytometry results. In all lanes, a lower-molecular-mass band of approximately 9–10 kDa was seen, faintly in the brain sample but more prominently in platelet and PBMC samples. As mAb 4F2 binds to an epitope spanning residues 61–76 (octarepeat region) of PrP, this could represent an N-terminal cleavage fragment of the protein.

To confirm the intracellular location of PrP C in platelets, both platelets and PBMCs were permeabilized and analysed by flow cytometry using mAbs 4F2, 8G8 and 6H4. PrP C was detected in both permeabilized platelets and permeabilized PBMCs (Fig. 1c). To confirm that permeabilization had occurred, staining with Alexa-fluor 568 phalloidin marker, which binds specifically to F-actin, was used as a positive control (data not shown). The platelets were contaminated with a small percentage of leukocytes (CD45 +), but these were excluded from the analysis by gating using light-scattering characteristics.

Influence of PrP genotype on PrP C expression by PBMCs

Susceptibility or resistance to scrapie in sheep is controlled to a large extent by coding polymorphisms of the PrP gene. The polymorphisms with the most significant effects are located at codons 136, 154 and 171, and alleles are designated by a three-letter code indicating the amino acids encoded at these positions. VRQ and ARQ alleles are associated with susceptibility to scrapie, whereas the ARR allele is associated with resistance. We examined the level of PrP C expression on PBMCs from healthy sheep of three PrP genotypes (VRQ/VRQ, ARQ/ARQ and ARR/ARR; n = 7 for each genotype) by indirect-immunofluorescence flow cytometry using mAbs FH11, 6H4 and 8G8, which bind to different epitopes on PrP, including the N-terminal and the more structured C-terminal domains of the protein. The MFI was calculated for each antibody as a measure of the amount of antibody bound to cell-surface PrP C (Fig. 2a). Regardless of the mAb used, PBMCs from VRQ/VRQ sheep expressed higher levels of PrP C than those from ARR-homozygous sheep, and these differences were statistically significant in pairwise comparisons using the Student’s t-test (P < 0.05). PBMCs from ARQ/ARQ sheep had a level of PrP C expression intermediate between those from VRQ/ VRQ and ARR/ARR sheep, but the differences were not statistically significant. All three mAbs produced a monophasic staining pattern in each PrP genotype (Fig. 2b), so the differences could not be explained by the biphasic staining pattern seen in ARQ and ARR homozygotes by other investigators using different antibodies (Thackray et al., 2004).

Level of PrP C expression on different lymphocyte subsets varies and is highest on a subpopulation of B cells

The level of PrP C expression on different PBMC subsets was examined by using two-colour indirect immunofluorescence on uninfected sheep with genotypes VRQ/VRQ and VRQ/ARQ (n = 4 for each genotype). The PrP-specific mAb 8G8 was used in combination with a panel of mAbs identifying different PBMC subsets. Comparison of the MFI calculated for each subset indicated that PrP C expression was detected at varying levels on all major subsets of PBMCs, including CD4 +, CD8 + and γδ T cells, B cells and monocytes (Fig. 3). Levels of expression showed a consistent pattern of variation, depending on cell type. There did not appear to be significant differences between VRQ/VRQ and VRQ/ARQ sheep in the amount of PrP C expressed on PBMC subsets, except in CD21 + and CD72 + B cells, where expression levels were much lower in VRQ/VRQ sheep. The reason for this difference is not clear. In both genotypes, high levels of PrP C expression were seen on CD62 L (L-selectin) cells, and PrP C was expressed at higher levels on CD21 + B cells than on CD72 + B cells.

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Expression of PrP on PBMCs of scrapie-infected sheep

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

DISCUSSION

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

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

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

Further work is required to determine whether PBMC subsets expressing higher levels of PrP<sup>C</sup> are more susceptible to scrapie infection and the implications for the peripheral pathogenesis of scrapie. However, a preliminary comparison of PrP expression levels in uninfected and scrapie-infected sheep suggested that, in some infected sheep, very high levels of PrP were found on CD21<sup>+</sup> B cells and CD62<sup>+</sup> lymphocytes. As we did not have access to mAbs that can distinguish PrP<sup>Sc</sup> from PrP<sup>C</sup>, it is not possible to say whether the apparent increase in protein levels is a result of...
PrP\textsuperscript{Sc} accumulation or upregulation of PrP\textsuperscript{C} expression in response to infection. Further investigations using larger numbers of scrapie-infected sheep will be necessary to establish whether this observation is consistent enough to demonstrate statistical significance and whether it reflects the distribution of infectivity.

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

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