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| 研究報告の概要 | <p>GPI (glycosylphosphatidylinositol) 結合型糖タンパクである PrP^C は、一般に PrP^{Sc} と呼ばれる疾患に関連するイソフォームへの構造変化を受け、感染性海綿状脳症 (TSE) の病因において中心的役割を果たす。PrP^C の正確な機能は明らかになっていないが、神経系以外の多くの組織で発現する。TSE が輸血によりヒツジに感染し得ることは、過去に示されている。本稿の目的は、感染因子を運ぶ血液成分を特定することであった。第一段階として、考えられる感染因子の標的を特定するため、ヒツジの血液細胞成分における PrP^C の分布を検討した。末梢血単核細胞 (PBMC) のみで細胞表面に PrP^C の発現が認められた。しかし、血小板にも有意な量の細胞内 PrP^C が認められた。PBMC の細胞表面に発現した PrP^C 値は PrP 遺伝子型の影響を受け、最高値はスクレイピー感受性 VRQ/VRQ ヒツジで、また最低値はスクレイピー耐性 ARR/ARR ヒツジで認められた。感受性ヒツジでは、PrP^C は PBMC の主なサブセットすべてにおいて異なるレベルで発現し、最高値は B 細胞 CD21(+)サブセットで認められた。CD21(+)B 細胞の PrP の発現が劇的に upregulate されたスクレイピー感染ヒツジにも存在した。</p> | | | | <p>使用上の注意記載状況・ その他参考事項等</p> <p>赤血球 M・A・P「日赤」 照射赤血球 M・A・P「日赤」</p> <p>血液を介するウイルス、 細菌、原虫等の感染 vCJD 等の伝播のリスク</p> |
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| 報告企業の意見 | | 今後の対応 | | | |
| <p>感染性海綿状脳症 (TSE) の感染因子を運ぶ血液成分を特定することを目的に、ヒツジの血液細胞成分における PrP^C の分布を研究したとの報告である。</p> | | <p>日本赤十字社では、vCJD の感染リスク低減の目的から、血液製剤の保存前白血球除去の導入を進めている。今後も引き続き、プリオン病に関する新たな知見及び情報の収集に努める。</p> | | | |

Expression of PrP^C on cellular components of sheep blood

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PrP^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^{Sc}. PrP^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^C on cellular components of sheep blood was examined to identify potential targets for infection. Cell-surface expression of PrP^C was found only on peripheral blood mononuclear cells (PBMCs); however, platelets also contained significant amounts of intracellular PrP^C. The level of PrP^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^C was expressed at varying levels on all major subsets of PBMCs, with the highest levels on the CD21⁺ subset of B cells, and PrP expression was upregulated dramatically on CD21⁺ B cells in some scrapie-infected sheep.

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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. Creutzfeldt–Jakob disease, CJD). A characteristic feature of these diseases is the accumulation of PrP^{Sc}, a post-translationally modified form of the host glycoprotein PrP^C, in the central nervous system (CNS). As PrP^{Sc} and infectivity co-purify (Bolton *et al.*, 1982), the presence of PrP^{Sc} is considered a marker for TSE infection. PrP^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^{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^{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^{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^{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^C 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^C. 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^C on blood cells (Barclay *et al.*, 2002; Holada & Vostal, 2000). In sheep, cell-surface expression of PrP^C 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^C 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^C in intracellular granules and may be responsible for release of soluble PrP^C into plasma (Perini *et al.*, 1996). The function of PrP^C in blood-associated cells is unknown, although experiments with PBMCs from humans 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^{Sc} from PrP^C 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^C and that the level of PrP^C expressed on PBMCs differed between scrapie-susceptible and -resistant sheep. We also demonstrated for the first time that PrP^C expression appears to be greatly upregulated on the CD21⁺ 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-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 VRQ/VRQ and VRQ/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⁻¹; Sigma) or Polymorphprep (Nycomed), with slight variations in the methods depending on the application.

Polymorphprep, a 1.11 g ml⁻¹ 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 1000 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⁻¹ (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 NH₄Cl, 10 mM KHCO₃, 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 5 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^C 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, IL-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.

Table 1. mAbs used for flow cytometry and Western blotting

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

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 ($1 \mu\text{g ml}^{-1}$), TD14 (1:10 dilution of culture supernatant) and CAPP2A ($5 \mu\text{g ml}^{-1}$) 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 ($1 \mu\text{g ml}^{-1}$) 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.

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 $2 \times$ 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 prepared in PBS containing 0.05% (v/v) Tween 20 (PBST). PrP was detected by probing the membrane with 4F2 [$1 \mu\text{g ml}^{-1}$ 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^C in cellular components of blood

Expression of PrP^C 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).

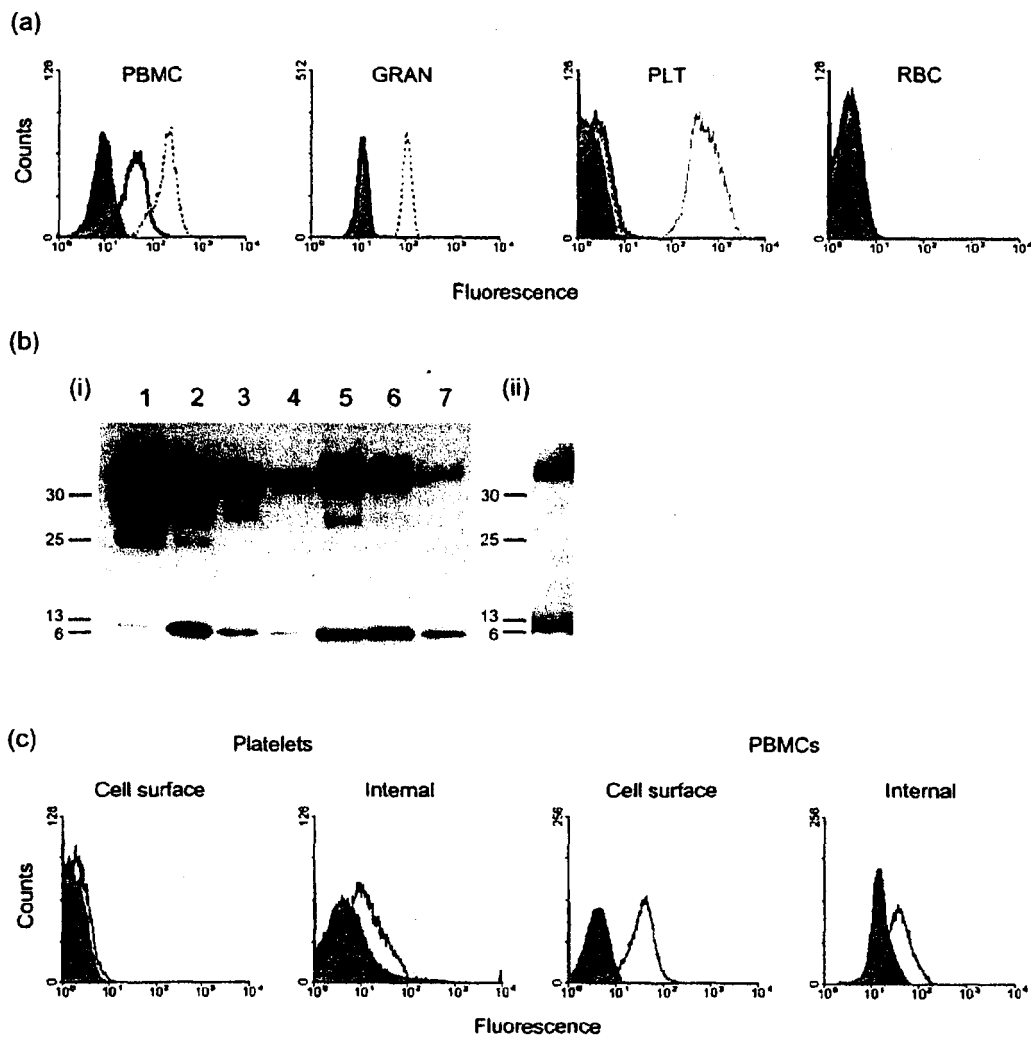


Fig. 1. Cellular distribution of PrP^C in uninfected sheep blood. (a) Cell-surface PrP^C expression on PBMCs, granulocytes (GRAN), platelets (PLT) and red blood cells (RBC) analysed by flow cytometry. Cells were stained by using antibodies to PrP (4F2, black line), CD45 (TD14, dashed line) and CD41/CD61 (CAPP2A, grey line). Filled area, negative controls (no antibodies). (b) Western blot analysis of PrP^C expression in sheep-blood cells, probed with mAb 4F2. (i) Lane 1, normal sheep-brain homogenate (equivalent to 80 ng tissue); lane 2, PBMCs ($\sim 1.6 \times 10^8$ cells); lanes 3 and 4, doubling dilutions of PBMCs; lane 5, platelets ($\sim 3.2 \times 10^8$ cells); lanes 6 and 7, doubling dilutions of platelets. Exposure time, 5 min. (ii) Red blood-cell membranes (equivalent to $\sim 1.6 \times 10^8$ cells). Exposure time, 4 h. (c) Flow-cytometry analysis of intracellular PrP^C 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. Polymorphprep 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 γ/δ 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-selectin) cells, and PrP^C was expressed at higher levels on CD21⁺ B cells than on CD72⁺ B cells.

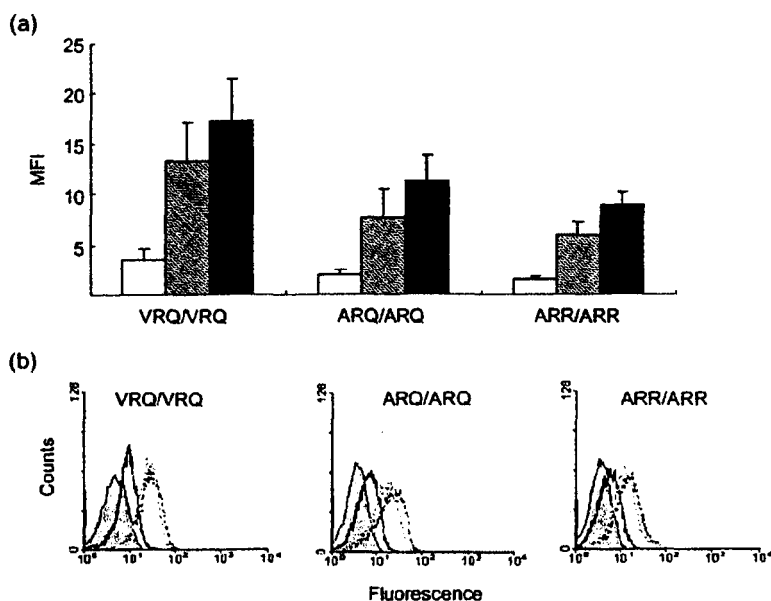


Fig. 2. Effect of PrP genotype on the PrP^C expression level of sheep PBMCs. (a) MFI values (mean ± SD; n = 7) are shown for PBMCs from uninfected sheep homozygous for VRQ, ARQ and ARR, incubated with FH11 (open bars), 6H4 (hatched bars) and 8G8 (filled bars). (b) Representative flow-cytometry staining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

Expression of PrP on PBMCs of scrapie-infected sheep

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

readily available, we could not tell whether the increase in cell-surface PrP was a result of accumulation of PrP^{Sc} or altered turnover of PrP^C.

DISCUSSION

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

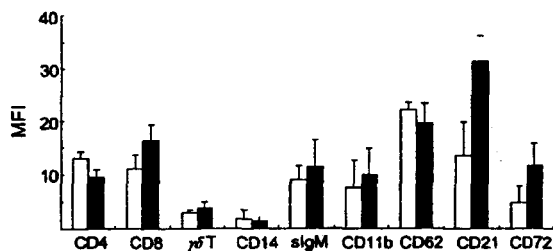


Fig. 3. PrP^C expression on PBMC subsets from uninfected sheep. PBMCs from VRQ/VRQ (open bars) and VRQ/ARQ (filled bars) sheep were analysed by two-colour immunofluorescent staining, using subset-specific mAbs and 8G8. Each bar represents the MFI (mean ± SD; n = 4) in the FL2 channel (PrP) for each subset.

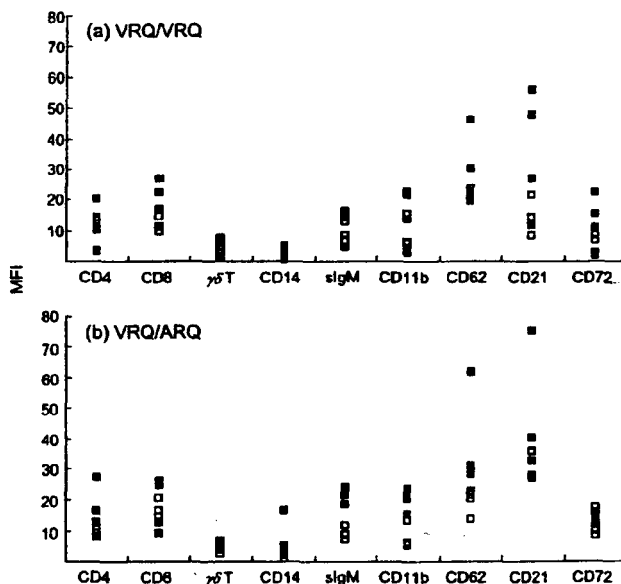


Fig. 4. Comparison of PrP^C expression on PBMC subsets from uninfected and scrapie-infected sheep. (a) VRQ/VRQ sheep; (b) VRQ/ARQ sheep. Individual values for MFI in the FL2 channel (PrP) are shown for uninfected (□) and scrapie-infected (■) sheep (n = 4 for each group).

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

PrP^C expression alone does not appear to be sufficient to render a particular tissue or cell type susceptible to TSE infection, as it is expressed in many tissues that do not become infected during disease (Bendheim *et al.*, 1992). Conversely, although hamster PBMCs do not express PrP^C, infectivity is associated with these cells in hamsters infected experimentally with scrapie (Holada *et al.*, 2002). In this case, it is possible that hamster PBMCs act as carriers of the infectious agent, without being actively infected themselves. Here, we have shown that the level of PrP^C expression on sheep PBMCs correlates with PrP genotype, with the highest levels found on PBMCs from sheep that are highly susceptible to scrapie (PrP genotype VRQ/VRQ) and the lowest levels on scrapie-resistant sheep (PrP genotype ARR/ARR). These results disagree with those published recently by Thackray *et al.* (2004), who concluded that PBMCs from scrapie-susceptible and -resistant sheep expressed similar levels of cell-surface PrP^C. The reason for the discrepancy is not clear, but probably relates to the different mAbs used in their experiments. They measured the binding of two N-terminal mAbs with predicted epitopes between residues 25 and 89 of sheep PrP and one of these mAbs did in fact stain a significantly higher percentage of PBMCs from VRQ-homozygous sheep compared with ARQ and ARR homozygotes. In our experiments, we used three PrP-specific mAbs with epitopes in different regions of the molecule at residues 54–57 (FH11), 95–110 (8G8) and 144–152 (6H4), which did not include the polymorphic residues at positions 136 and 171. The genotype-specific differences in PrP^C staining were observed consistently with each of these antibodies, making it unlikely that the results can be explained by differences in antibody affinity for the variant PrP proteins or subtle differences in protein conformation that might influence the accessibility of certain epitopes to antibody binding. The much lower levels of binding of FH11 compared with the other two mAbs may possibly be explained by expression of an N-terminally truncated form of PrP^C on PBMCs, which would lack the

FH11 epitope. Further work will be required to determine whether PrP-dependent differences in PrP^C expression levels extend to other tissues and to elucidate the mechanisms controlling protein levels expressed by different PrP alleles. These could operate at the level of gene transcription and translation, or result from differences in the rate of turnover of different PrP proteins. Thermal-denaturation studies with recombinant ovine PrP proteins have shown that the formation of unfolding intermediates of VRQ and ARQ proteins requires higher activation energy than in the case of ARR proteins, implying that susceptibility-associated PrP variants are intrinsically more stable than the ARR protein (Rezaei *et al.*, 2002). In addition, it has been found that recombinant ARR protein is degraded more rapidly by proteasomes than VRQ protein (Tenzer *et al.*, 2004). Factors such as these might contribute to a more rapid turnover of ARR PrP, leading to reduced cell-surface levels. It is possible to speculate that this could contribute to disease resistance by reducing the opportunity for conversion of ARR protein to the disease-associated isoform.

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

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

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

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

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REFERENCES

- Barclay, G. R., Hope, J., Birkett, C. R. & Turner, M. L. (1999). Distribution of cell-associated prion protein in normal adult blood determined by flow cytometry. *Br J Haematol* 107, 804–814.
- Barclay, G. R., Houston, E. F., Halliday, S. I., Farquhar, C. F. & Turner, M. L. (2002). Comparative analysis of normal prion protein expression on human, rodent, and ruminant blood cells by using a panel of prion antibodies. *Transfusion* 42, 517–526.
- Bembridge, G. P., Howard, C. J., Parsons, K. R. & Sopp, P. (1993). Identification of monoclonal antibodies specific for bovine leukocyte common antigen (CD45) together with a novel broadly expressed leukocyte differentiation antigen, BoWC11. *Vet Immunol Immunopathol* 39, 115–120.
- Bendheim, P. E., Brown, H. R., Rudelli, R. D., Scala, L. J., Goller, N. L., Wen, G. Y., Kascsak, R. J., Cashman, N. R. & Bolton, D. C. (1992). Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. *Neurology* 42, 149–156.
- Blättler, T., Brandner, S., Raeber, A. J., Klein, M. A., Voigtländer, T., Weissmann, C. & Aguzzi, A. (1997). PrP-expressing tissue required for transfer of scrapie infectivity from spleen to brain. *Nature* 389, 69–73.
- Bolton, D. C., McKinley, M. P. & Prusiner, S. B. (1982). Identification of a protein that purifies with the scrapie prion. *Science* 218, 1309–1311.
- Brown, P. (1995). Can Creutzfeldt-Jakob disease be transmitted by transfusion? *Curr Opin Hematol* 2, 472–477.
- Brown, P., Rohwer, R. G., Dunstan, B. C., MacAuley, C., Gajdusek, D. C. & Drohan, W. N. (1998). The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. *Transfusion* 38, 810–816.
- Brown, P., Cervenakova, L., McShane, L. M., Barber, P., Rubenstein, R. & Drohan, W. N. (1999). Further studies of blood infectivity in an experimental model of transmissible spongiform encephalopathy, with an explanation of why blood components do not transmit Creutzfeldt-Jakob disease in humans. *Transfusion* 39, 1169–1178.
- Bruce, M. E., Brown, K. L., Mabbott, N. A., Farquhar, C. F. & Jeffrey, M. (2000). Follicular dendritic cells in TSE pathogenesis. *Immunol Today* 21, 442–446.
- Cashman, N. R., Loertscher, R., Nalbantoglu, J., Shaw, I., Kascsak, R. J., Bolton, D. C. & Bendheim, P. E. (1990). Cellular isoform of the scrapie agent protein participates in lymphocyte activation. *Cell* 61, 185–192.
- Eklund, C. M., Kennedy, R. C. & Hadlow, W. J. (1967). Pathogenesis of scrapie virus infection in the mouse. *J Infect Dis* 117, 15–22.
- Gupta, V. K., McConnell, I., Dalziel, R. G. & Hopkins, J. (1998). Two B cell subpopulations have distinct recirculation characteristics. *Eur J Immunol* 28, 1597–1603.
- Hadlow, W. J., Kennedy, R. C. & Race, R. E. (1982). Natural infection of Suffolk sheep with scrapie virus. *J Infect Dis* 146, 657–664.
- Hall, G. A., Sopp, P. & Howard, C. J. (1993). An investigation of temporary workshop clusters reacting with cells of the mononuclear phagocytic system. *Vet Immunol Immunopathol* 39, 225–236.
- Herrmann, L. M., Davis, W. C., Knowles, D. P., Wardrop, K. J., Sy, M. S., Gambetti, P. & O'Rourke, K. I. (2001). Cellular prion protein is expressed on peripheral blood mononuclear cells but not platelets of normal and scrapie-infected sheep. *Haematologica* 86, 146–153.
- Herrmann, L. M., Baszler, T. V., Knowles, D. P. & Cheevers, W. P. (2002). PrP^{Sc} is not detected in peripheral blood leukocytes of scrapie-infected sheep: determining the limit of sensitivity by immunohistochemistry. *Clin Diagn Lab Immunol* 9, 499–502.
- Hill, A. F., Butterworth, R. J., Joiner, S. & 12 other authors (1999). Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. *Lancet* 353, 183–189.
- Holada, K. & Vostal, J. G. (2000). Different levels of prion protein (PrP^C) expression on hamster, mouse and human blood cells. *Br J Haematol* 110, 472–480.
- Holada, K., Mondoro, T. H., Muller, J. & Vostal, J. G. (1998). Increased expression of phosphatidylinositol-specific phospholipase C resistant prion proteins on the surface of activated platelets. *Br J Haematol* 103, 276–282.
- Holada, K., Vostal, J. G., Theisen, P. W., MacAuley, C., Gregori, L. & Rohwer, R. G. (2002). Scrapie infectivity in hamster blood is not associated with platelets. *J Virol* 76, 4649–4650.
- Houston, F., Foster, J. D., Chong, A., Hunter, N. & Bostock, C. J. (2000). Transmission of BSE by blood transfusion in sheep. *Lancet* 356, 999–1000.
- Houston, E. F., Halliday, S. I., Jeffrey, M., Goldmann, W. & Hunter, N. (2002). New Zealand sheep with scrapie-susceptible PrP genotypes

- succumb to experimental challenge with a sheep-passaged scrapie isolate (SSBP/1). *J Gen Virol* 83, 1247–1250.
- Howard, C. J., Sopp, P. & Parsons, K. R. (1992). L-selectin expression differentiates T cells isolated from different lymphoid tissues in cattle but does not correlate with memory. *Immunology* 77, 228–234.
- Hunter, N., Foster, J., Chong, A., McCutcheon, S., Parnham, D., Eaton, S., MacKenzie, C. & Houston, F. (2002). Transmission of prion diseases by blood transfusion. *J Gen Virol* 83, 2897–2905.
- Korh , C., Stierli, B., Streit, P. & 14 other authors (1997). Prion (PrP^{Sc})-specific epitope defined by a monoclonal antibody. *Nature* 390, 74–77.
- Krasemann, S., Jurgens, T. & Bodemer, W. (1999). Generation of monoclonal antibodies against prion proteins with an unconventional nucleic acid-based immunization strategy. *J Biotechnol* 73, 119–129.
- Llewelyn, C. A., Hewitt, P. E., Knight, R. S. G., Amar, K., Cousens, S., Mackenzie, J. & Will, R. G. (2004). Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet* 363, 417–421.
- Mabbott, N. A., Brown, K. L., Manson, J. & Bruce, M. E. (1997). T-lymphocyte activation and the cellular form of the prion protein. *Immunology* 92, 161–165.
- MacGregor, I., Hope, J., Barnard, G. & 8 other authors (1999). Application of a time-resolved fluoroimmunoassay for the analysis of normal prion protein in human blood and its components. *Vox Sang* 77, 88–96.
- MacHugh, N. D. & Sopp, P. (1991). Individual antigens of cattle. Bovine CD8 (BoCD8). *Vet Immunol Immunopathol* 27, 65–69.
- Mackay, C. R., Beya, M. F. & Matzinger, P. (1989). Gamma/delta T cells express a unique surface molecule appearing late during thymic development. *Eur J Immunol* 19, 1477–1483.
- Maddox, J. F., Mackay, C. R. & Brandon, M. R. (1985). Surface antigens, SBU-T4 and SBU-T8, of sheep T lymphocyte subsets defined by monoclonal antibodies. *Immunology* 55, 739–748.
- Maddox, J. F., Mackay, C. R. & Brandon, M. R. (1987). Ontogeny of ovine lymphocytes. II. An immunohistological study on the development of T lymphocytes in the sheep fetal spleen. *Immunology* 62, 107–112.
- Mateo, A., Pintado, C. O., Perez de la Lastra, J., Dusinsky, R., Simon, M., Naessens, J. & Llanes, D. (1996). Ruminant cluster CD41/CD61. *Vet Immunol Immunopathol* 52, 251–253.
- Peden, A. H., Head, M. W., Ritchie, D. L., Bell, J. E. & Ironside, J. W. (2004). Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* 364, 527–529.
- Perini, F., Vidal, R., Ghetti, B., Tagliavini, F., Frangione, B. & Prelli, F. (1996). PrP^{27–30} is a normal soluble prion protein fragment released by human platelets. *Biochem Biophys Res Commun* 223, 572–577.
- Prusiner, S. B. (1998). Prions. *Proc Natl Acad Sci U S A* 95, 13363–13383.
- Rezaei, H., Choiset, Y., Eghlalian, F., Treguer, E., Mentre, P., Debey, P., Grosclaude, J. & Haertle, T. (2002). Amyloidogenic unfolding intermediates differentiate sheep prion protein variants. *J Mol Biol* 322, 799–814.
- Schmerr, M. J., Jenny, A. L., Bulgin, M. S., Miller, J. M., Hamr, A. N., Cutlip, R. C. & Goodwin, K. R. (1999). Use of capillary electrophoresis and fluorescent labeled peptides to detect the abnormal prion protein in the blood of animals that are infected with a transmissible spongiform encephalopathy. *J Chromatogr A* 853, 207–214.
- Sopp, P. (1996). Ruminant cluster CD21. *Vet Immunol Immunopathol* 52, 249.
- Sopp, P. & Howard, C. J. (1997). Cross-reactivity of monoclonal antibodies to defined human leucocyte differentiation antigens with bovine cells. *Vet Immunol Immunopathol* 56, 11–25.
- Sopp, P., Kwong, L. S. & Howard, C. J. (1996). Identification of bovine CD14. *Vet Immunol Immunopathol* 52, 323–328.
- Tenzer, S., Stoltze, L., Sch nfisch, B., Dengjel, J., M ller, M., Stevanovi , S., Rammensee, H.-G. & Schild, H. (2004). Quantitative analysis of prion-protein degradation by constitutive and immunosomosomes indicates differences correlated with disease susceptibility. *J Immunol* 172, 1083–1091.
- Thackray, A. M., Yang, S., Wong, E., Fitzmaurice, T. J., Morgan-Warren, R. J. & Bujdoso, R. (2004). Conformational variation between allelic variants of cell-surface ovine prion protein. *Biochem J* 381, 221–229.
- Wadsworth, J. D. F., Joiner, S., Hill, A. F., Campbell, T. A., Desbruslais, M., Luthert, P. J. & Collinge, J. (2001). Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. *Lancet* 358, 171–180.
- Young, A. J., Marston, W. L., Dessing, M., Dudler, L. & Hein, W. R. (1997). Distinct recirculating and non-recirculating B-lymphocyte pools in the peripheral blood are defined by coordinated expression of CD21 and L-selectin. *Blood* 90, 4865–4875.