the observed numbers of cases, estimates of the French exposure are only involved in the description by gender, age, and calendar year of simulated vCJD cases.

We set the end of the exposure period in 1995 as the embargo on British beef was ordered at the beginning of 1996. Afterwards, indigenous BSE constituted the unique source of infection of the French population. A total of 1500 French bovine carcasses were estimated to have entered the human food chain after 1995.[9] If all those carcasses had been used to produce MRM, French exposure between 1996 and 2001 would have represented less than 2% of the French total exposure.

Other limitations of these predictive models have been already pointed out. Other genotypes at the *PRNP* gene codon 129 could be susceptible to the BSE agent with longer incubation period. Indeed, in both iatrogenic CJD due to human growth hormone treatment[17] and Kuru,[18,19] individuals with the methionine-valine heterozygous genotype, which represents about 50% of the French population, have longer incubation periods than the methionine-methionine homozygotes. As heterozygotes also have a lower susceptibility to prion diseases, they should not contribute much to the vCJD epidemics. Moreover, possible transmission of vCJD by blood *transfusion was suggested by recent case reports in the UK*[20,21] and iatrogenic transmission of vCJD through medical or surgical procedures cannot be excluded. But, a series of effective measures to reduce the risk of transmission of vCJD by infected material and blood products were taken in France. In addition, transfused individuals were banned from blood donation.

Predictions of the vCJD epidemics in the UK, France, and the Republic of Ireland[6] are consistent and reassuring. To date, the best estimates of the number of future clinical cases were between 200 and 400 cases in the UK, approximately 30 in France and between one and two in the Republic of Ireland. *The Republic of Ireland had the second highest incidence of BSE* worldwide. Harney *et al.* estimated that exposure due to the BSE epidemic in Ireland and exposure due to Irish imports from the UK were equivalent.[6] Our study suggests that, in France, imports from the UK have represented the main source of infection by the BSE agent and that exposure due to BSE in French cattle plays a negligible role in the vCJD epidemic.

Data from Customs and Excise in the UK indicated that, over the period 1980–1995, about 60% of the total exports of UK bovine carcasses to the European Community (EC) countries (about 2 million tonnes equivalent of carcasses) were exported to France. Therefore, very few vCJD cases due to the past BSE epidemics are expected in other EC countries, and worldwide. Nevertheless, as long as BSE and other forms of animal transmissible spongiform encephalopathies are not eliminated, surveillance of human prion diseases at both the national and international levels remains necessary.

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KEY MESSAGES

- The French population may have been mainly exposed to the BSE agent through the consumption of BSE-infected bovines which were imported from the UK.
- Thirty-three future vCJD cases are expected in the French population, with the upper bound at lower than 100 cases.
- Expected cases of vCJD are young: two-thirds of the simulated vCJD cases are expected in the post-1969 birth-cohort and the remaining one-third in the 1940–69 cohort. Cases in people born before 1939 are very unlikely to occur.
- No gender-related susceptibility to the BSE agent can be outlined.

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Commentary: The risk of variant Creutzfeldt–Jakob Disease: reassurance and uncertainty

RG Will

The annual number of deaths from variant Creutzfeldt–Jakob Disease (vCJD) in the UK is currently on a decline.[1] Epidemiological and laboratory evidence strongly supports the hypothesis that vCJD is caused by human infection with bovine spongiform encephalopathy (BSE) and the population risk of developing this condition is likely to be proportional to the extent of human exposure to BSE, presumptively through contaminated meat products. The risk of vCJD in countries other than the UK may be due to exposure to indigenous BSE, import of infected animals, animal feed, and food products from the UK, or exposure to BSE during travel to the UK in the risk period 1980–1996. The paper by Chadeau-Hyam and Alperovitch[2] assesses these potential risks in France and concludes that overall there may be a limited number of future vCJD cases in the French population (33 cases from 2004–2020) and that the main risk was through consumption of infected bovines from the UK. Travel to the UK was assessed to account for only 2% of BSE exposure and exposure to French cases of BSE was not considered because this was judged to represent a low risk. This paper and a similar study in Ireland[3] suggest that the number of future cases of vCJD may be very limited outside the UK. There are, however, a number of important caveats.

To date all clinical cases of vCJD in which the prion protein gene (*PRNP*) has been examined have been methionine homozygotes, with no identified cases in the 68% of the Caucasian population with the alternative valine homozygotes or heterozygous genotypes. All predictive studies of vCJD to date have overtly assumed that only methionine homozygotes will be affected, but the possibility that infection with BSE can occur in the other genetic backgrounds has been supported by the recent publication of a presumed preclinical† case of vCJD in a *PRNP* heterozygous blood transfusion recipient.[4] If heterozygotes can be infected with BSE it would be surprising if valine homozygotes could not also be infected, although Chadeau-Hyam and Alperovitch suggest that heterozygotes (and presumably valine homozygotes) may have a lower susceptibility to infection and may not add significantly to the vCJD epidemic. Cattle are uniformly methionine homozygotes and homology of prion protein types is thought to lead to

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†The possibility of life-long infection without the development of disease cannot be excluded.

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研究報告の概要

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。

使用上の注意記載状況・その他参考事項等

赤血球M・A・P「日赤」
照射赤血球 M・A・P「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD 等の伝播のリスク

報告企業の意見	今後の対応
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。	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。

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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. Creutzfeld–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 NH_4Cl, 10 mM $KHCO_3$, 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 20 000 *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 *et al.* (1999)
8G8	IgG2a	PrP	95–110	Krasemann *et al.* (1999)
6H4	IgG1	PrP	144–152	Korth *et al.* (1997)
44-97	IgG1	CD4 (T-cell subset)	–	Maddox *et al.* (1985)
IL-A51	IgG1	CD8 (T-cell subset)	–	MacHugh & Sopp (1991)
86D	IgG1	γ/δ T-cell receptor	–	Mackay *et al.* (1989)
CC-G33	IgG1	CD14 (monocytes)	–	Sopp *et al.* (1996)
25-69	IgG1	sIgM (B cells)	–	Maddox *et al.* (1987)
CC125	IgG1	CD11b (B-cell subset)	–	Hall *et al.* (1993)
CC32	IgG1	CD62 (lymphocyte subsets)	–	Howard *et al.* (1992)
CC21	IgG1	CD21 (B cells)	–	Sopp (1996)
DU2-104	IgM	CD72 (B cells)	–	Young *et al.* (1997)
CAPP2A	IgG1	CD41/CD61 (platelets)	–	Mateo *et al.* (1996)
TD14	IgG1	CD45 (leukocytes)	–	Bembridge *et al.* (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 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.

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 prepared 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^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).

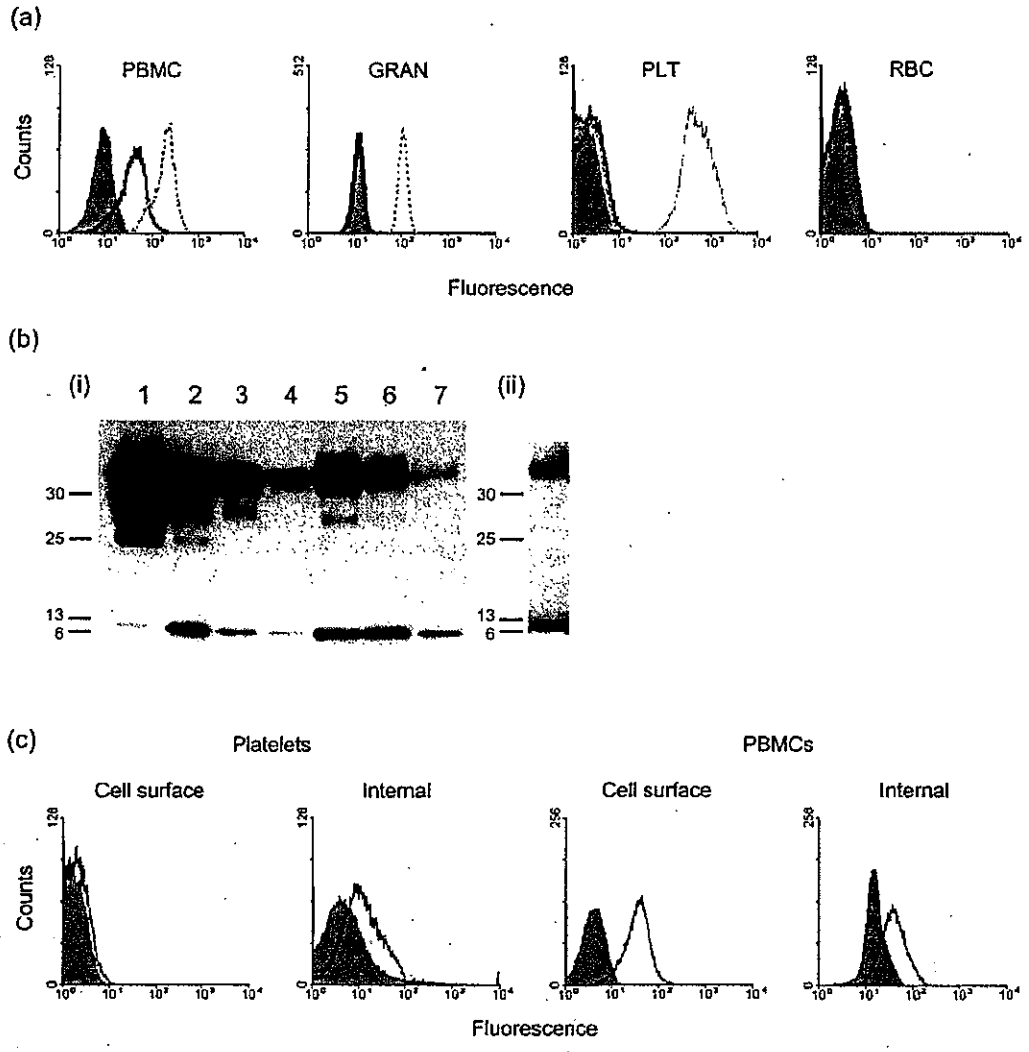

Fig. 1. Cellular distribution of PrP^C in uninfected sheep blood. (a) Cell-surface PrP^C expression on PBMCs, granulocytes (GRAN), platelets (PLT) and red blood cells (RBC) analysed by flow cytometry. Cells were stained by using antibodies to PrP (4F2, black line), CD45 (TD14, dashed line) and CD41/CD61 (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 ($\sim 1{\cdot}6 \times 10^6$ cells); lanes 3 and 4, doubling dilutions of PBMCs; lane 5, platelets ($\sim 3{\cdot}2 \times 10^8$ cells); lanes 6 and 7, doubling dilutions of platelets. Exposure time, 5 min. (ii) Red blood-cell membranes (equivalent to $\sim 1{\cdot}6 \times 10^8$ 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 ($P<0{\cdot}05$). 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.