Detection of PrP^{sc} in Blood from Sheep Infected with the Scrapie and Bovine Spongiform Encephalopathy Agents[∇]

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The role of blood in the iatrogenic transmission of transmissible spongiform encephalopathy (TSE) or prion disease has become an increasing concern since the reports of variant Creutzfeldt-Jakob disease (vCJD) transmission through blood transfusion from humans with subclinical infection. The development of highly sensitive rapid assays to screen for prion infection in blood is of high priority in order to facilitate the prevention of transmission via blood and blood products. In the present study we show that PrP^{sc}, a surrogate marker for TSE infection, can be detected in cells isolated from the blood from naturally and experimentally infected sheep by using a rapid ligand-based immunoassay. In sheep with clinical disease, PrP^{sc} was detected in the blood of 55% of scrapie agent-infected animals ($n = 80$) and 71% of animals with bovine spongiform encephalopathy ($n = 7$). PrP^{sc} was also detected several months before the onset of clinical signs in a subset of scrapie agent-infected sheep, followed from 3 months of age to clinical disease. This study confirms that PrP^{sc} is associated with the cellular component of blood and can be detected in preclinical sheep by an immunoassay in the absence of in vitro or in vivo amplification.

Transmission of variant Creutzfeldt-Jakob disease (vCJD) has been linked with blood transfusion in four reported cases in Great Britain (19, 24, 26, 32), indicating that this is likely to be an efficient route of transmission. Such findings highlight a significant risk to recipients of vCJD-contaminated blood components, and blood services in the United Kingdom have responded by putting in place precautionary measures, including leucodepletion. However, it remains uncertain whether such a procedure is able to remove all prion infectivity. For example, in two studies by Gregori et al. (13, 14) only 42 and 72% of infectivity was removed by leucodepletion from blood from hamsters with scrapie. Therefore, a rapid blood test for vCJD that is able to screen for likely infected blood is critical given that the presymptomatic stages of vCJD are long and that the prevalence of infection in the human population is unknown (6, 9). This knowledge has given rise to concerns that a large-scale vCJD epidemic could occur by human-to-human transmission (16, 21).

Infectivity in human blood is consistent with the demonstration of transmission of disease by blood transfusion in sheep incubating both scrapie and experimental BSE infection (17, 18, 20). Transmission was demonstrated from both whole blood and buffy coat fractions from sheep blood, indicating a cellular source of prions although, from studies done in rodent models, it is likely that the plasma fraction also contains infectivity (4, 13, 14). Furthermore, transmission was possible from sheep showing clinical signs and from sheep that were infected

but still in the preclinical phase. However, identification of the abnormal prion protein (PrP^{sc}) in blood as a surrogate marker for infection has proved more elusive (3). Recently, PrP^{sc} has been amplified from the blood of experimentally infected rodents (5, 25, 28) and from sheep naturally infected with scrapie agent (29) using protein misfolded cyclic amplification (PMCA), but often these studies take days or weeks to complete. Here, we demonstrate, using a ligand-based immunoassay, that PrP^{sc} is associated with blood leukocytes from sheep with terminal scrapie or bovine spongiform encephalopathy (BSE) and in sheep incubating scrapie prior to the onset of clinical signs. This assay is a modification of a test that has been validated for use as a postmortem test for BSE, scrapie, and chronic wasting disease (CWD) in Europe and the United States (7).

MATERIALS AND METHODS

Sheep sampling and animal details. All animal procedures were approved by the home office under the Animals (Scientific Procedures) Act 1986. Peripheral blood was collected by jugular venipuncture from sheep with scrapie or BSE or from uninfected control sheep.

Blood from sheep naturally infected with scrapie agent was obtained from animals bred in a flock with 100% incidence of scrapie in sheep carrying the scrapie-susceptible VRQ/VRQ genotype. This flock was stocked predominantly with sheep carrying scrapie-susceptible PrP genotypes from flocks with known scrapie: the first confirmed case of scrapie in this flock occurred in 1995. The flock has since been maintained by a breeding program designed to maintain disease susceptibility and also by introducing new susceptible sheep in the following years from various farm sources. It is likely therefore that there is a mixture of prion strains in this flock. Clinical scrapie occurred in the sheep tested at a mean of 687 days after birth in the VRQ/VRQ sheep ($n = 56$; standard deviation [SD] = 59 days; range, 502 to 843 days) and 827 days in ARQ/VRQ sheep ($n = 19$; SD = 78 days; range, 742 to 944 days). Other genotypes tested were four ARR/VRQ animals and one ARH/VRQ animal for which the data are incomplete.

Seven sheep with BSE at clinical endpoint (mean incubation period [i.e., the

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time between inoculation and euthanasia due to clinical disease] of 658 days) provided a second source of blood. These sheep, all with ARQ/ARQ PrP genotypes, were challenged orally with 2.5 g of a pooled inoculum created from the brainstems of 20 histologically confirmed clinical cases of cattle with BSE. The brain pool was stored at -70°C prior to oral inoculation as a 10% saline suspension. Considerable variation in incubation periods has been reported for individual sheep with BSE postinoculation, ranging from 628 to 1,132 days (2).

Control blood from uninfected, age- and genotype-matched animals ($n = 129$) was obtained from a closed breeding flock that had been originally stocked with animals imported directly from New Zealand and maintained free of classical scrapie by strict biosecurity measures.

To determine whether PrP^{Sc} could be detected in the blood of sheep with preclinical disease, we monitored PrP^{Sc} levels in blood from sheep ($n = 11$ VRQ/VRQ, $n = 11$ ARQ/VRQ, and $n = 1$ ARR/VRQ animals) in the Veterinary Laboratories Agency (VLA) flock during an 18-month period. Due to time constraints, we were unable to monitor sheep from birth to clinical endpoint, so we selected sheep from different birth cohorts (2006, 2007, and 2008) in order to cover the entire incubation period.

Sheep were sedated prior to blood sampling at the clinical endpoint. All blood samples from the scrapie agent-infected sheep and matched uninfected controls were processed immediately, usually within 4 h of sampling. Samples from both sheep with BSE and matched uninfected control sheep were stored overnight at 4°C prior to sample processing.

RAMALT biopsy. All VRQ/VRQ sheep in the flock with endemic scrapie accumulate PrP^{Sc} in lymphoid tissue from 2 to 4 months of age (S. J. Bellworthy, unpublished data). However, the incidence of scrapie infection in the ARQ/VRQ sheep is $<100\%$; so, to determine whether live sheep were infected, rectoanal mucosa-associated lymphoid tissue (RAMALT) samples were obtained from ARQ/VRQ sheep at 26 months of age from the sheep in the time course study. This method is a premortem test that is able to confirm infection in a high proportion of sheep with scrapie agent (11), but this is dependent on genotype and age. Also, RAMALT may accumulate PrP^{Sc} later in the incubation period than other lymphoid tissues, e.g., the distal ileum (11, 22), and so cannot be relied upon as an absolute marker for lymphoid tissue involvement in transmissible spongiform encephalopathies (TSEs). Immunohistochemical examination of fixed premortem RAMALT samples was undertaken using antibody R145 (10).

Blood preparation and processing. Briefly, EDTA-treated blood was centrifuged in Vacutainers at $670 \times g$ for 30 min at 21°C to isolate the buffy coat. Buffy coats were diluted in wash buffer (Dulbecco phosphate-buffered saline containing 0.002% EDTA and 0.1% bovine serum albumin), and the cell suspension was layered over Histopaque (1.077; Sigma-Aldrich) and centrifuged at $800 \times g$ for 50 min at 20°C . Peripheral blood mononuclear cells (PBMC) were recovered from the interface and washed in phosphate-buffered saline prior to counting. Aliquots of 10^7 cells from ~ 5 ml of whole blood were frozen without buffer at -80°C until used.

Lymph node dissociation and titration. Lymph nodes from VRQ/VRQ and ARQ/VRQ sheep with terminal scrapie were removed at postmortem, and the cells were dissociated mechanically. Cells were serially diluted (3×10^4 to 3×10^6) into the wells of a ligand-based immunoassay. Values were plotted against cell number, and the minimum number of cells required to give an absorbance value of ≥ 0.056 was calculated by transformation and linear regression ($r = 0.97$), followed by extrapolation and interpolation (GraphPad Prism).

Ligand-based immunoassay. The assay is a modified form of the IDEXX HerdChek assay developed for CWD and licensed in the United States for the detection of PrP^{Sc} in lymphoid tissue from deer infected with CWD. A similar assay is licensed in Europe for scrapie and BSE detection in brain and has been validated (7). Both assays depend on polyanionic ligand binding (23) with subsequent detection of PrP by specific antibodies. Detection of PrP^{Sc} and specificity is realized by a combination of buffer components in the diluent that eliminate PrP^C binding. Increased sensitivity is achieved with a mild trypsin digestion, although the trypsin is not required for specificity. The number of PBMC in the assay was set at a constant of 10^7 , and the signal in the immunoassay ranged from below the levels of detection (an absorbance of <0.045) up to an absorbance of 0.35. We have modified this assay (12) to detect PrP^{Sc} in RAMALT from scrapie agent-infected sheep. Proteinase K is not used in the assay. Briefly, PBMC at 5×10^7 , the equivalent of 25 ml of blood, were thawed prior to use and then transferred to the ribolysate tube. Two cycles of ribolysis were performed, cooling to 4°C between cycles. Cell homogenate was transferred to a fresh tube, and working plate diluent added; this suspension was then transferred to the assay wells of the coated plate. Incubation was for 2.5 h at 21°C with constant shaking. After further washing and the application of a conditioning buffer, bound PrP^{Sc} was detected by peroxidase-conjugated PrP antibody and visualization with tetramethyl benzidine substrate. All samples were evaluated in duplicate or tripli-

FIG. 1. Ligand-based immunoassay that detects PrP^{Sc} associated with PBMC from TSE agent-infected sheep at the clinical endpoint. The equivalent of ten million lysed cells were assayed per well in duplicate or triplicate (standard error, $<5\%$). The means of the actual absorbance (450 to 620 nm) values from individual sheep are shown; one symbol represents one sheep. The horizontal lines represent the means of each group. Genotype-matched control sheep ($n = 129$) were obtained from the VLA scrapie-free flock. For the column marked ARX/VRQ, the symbols represent four ARR/VRQ and one ARH/VRQ scrapie agent-infected sheep. These have been grouped together for simplicity, and the two lowest values were obtained for two of the ARR/VRQ sheep. A significant difference ($P < 0.01$) is observed for the means of the VRQ/VRQ values compared to the mean of the controls (analysis of variance).

cate depending on size of original blood sample. All plates contained assay controls, as provided by the manufacturer and, in addition, PBMC from 10 to 15 uninfected control sheep were assayed to calculate the cutoff value. Absorbance was read at 450 and 620 nm using a Victor multiwell plate reader (Perkin-Elmer) and the difference calculated to provide the final optical density. The uninfected control sheep were genotype matched to the genotype of the infected sheep. The cutoff value was calculated as the mean plus three times the SDs of the optical densities recorded for the negative control samples. This value was set at 0.056 after 129 individual uninfected control samples had been assayed on several days, using several batches of the HerdChek kit.

To normalize for variation from assay to assay, the values for the time course study have been normalized by subtraction of the means of 10 individual negative controls included in each plate. The cutoff points have been calculated as three SDs of the combined negative controls of the relevant assay plates.

RESULTS AND DISCUSSION

Detection of PrP^{Sc} in sheep with clinical scrapie or BSE.

Blood was collected from sheep showing definitive clinical signs of scrapie ($n = 80$) or BSE ($n = 7$) and disease was subsequently confirmed by the presence of PrP^{Sc}, as shown by both Western blotting and immunohistochemistry, in the brain at postmortem.

In scrapie agent-infected sheep, PrP^{Sc} was detected in the PBMC from 33 of 56 VRQ/VRQ sheep (59%), 8 of 19 ARQ/VRQ sheep (42%), and 3 of 5 sheep (60%) with other PrP genotypes (Fig. 1), all of which were at the clinical endpoint.

In ARQ/ARQ sheep with BSE, PrP^{Sc} was detected in five of seven animals (71%, Fig. 1), indicating that PrP^{Sc} also circulates in the PBMC of sheep experimentally infected with BSE agent.

These data show that PrP^{Sc} can be detected in the blood of sheep with scrapie and BSE and are consistent with the demonstration that infectivity resides in the blood (17, 18, 20). This assay is the first to demonstrate PrP^{Sc} in blood by a direct immunoassay that does not require *in vitro* amplification. The

Sheep ID	Genotype	TSE Status	Age at death (months)	Birth cohort	Age sampled (months)																													
					3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		
Sh1	VRQ/VRQ	A		2008	-	-	-	-	-																									
Sh2	VRQ/VRQ	A		2007	-	-	-	-	-	+	-	-	-	+	+																			
Sh3	VRQ/VRQ	A		2007	-	-	-	-	+	+	-	+	-	-	-																			
Sh4	VRQ/VRQ	A		2007	-	-	-	-	-	-	-	-	-	+	-																			
Sh5	VRQ/VRQ	A		2007	-	-	-	-	-	-	-	-	-	-	-																			
Sh6	VRQ/VRQ	A		2007	-	-	-	-	-	-	-	-	-	-	-																			
Sh13	VRQ/VRQ	pos	22	2006																														
Sh14	VRQ/VRQ	pos	21	2006																														
Sh15	VRQ/VRQ	pos	17	2006																														
Sh16	VRQ/VRQ	pos	21	2006																														
Sh17	VRQ/VRQ	pos	21	2006																														
Sh7	ARQ/VRQ	A		2007		-	+	-	-	-	+	-	-	+																				
Sh8	ARQ/VRQ	A		2007		-	-	-	-	-	-	-	-	-																				
Sh9	ARQ/VRQ	A		2008	-	-	-	-	-																									
Sh10	ARQ/VRQ	A		2008	-	-	-	-	-																									
Sh11	ARQ/VRQ	A		2008	-	-	-	-	-																									
Sh12	ARQ/VRQ	A		2008	-	-	-	-	-																									
Sh18	ARQ/VRQ	pos	28	2006																														
Sh19	ARQ/VRQ	pos	30	2006																														
Sh20	ARQ/VRQ	pos	35	2006																														
Sh21	ARQ/VRQ	pos	32	2006																														
Sh22	ARQ/VRQ	A		2006																														
Sh23	ARR/VRQ	A		2006

RAMALT biopsy
Not tested
 + blood positive
 - blood negative
 A alive at time of writing
 pos confirmed scrapie positive at post mortem

FIG. 2. Details of sheep in the time course study. Detection of PrP^{sc} in PBMC from sheep exposed to scrapie but not showing clinical signs. Samples were analyzed monthly from sheep born into a flock with endemic scrapie. RAMALT biopsy samples were taken at 26 months from selected sheep as shown, and the results are indicated in Table 1.

results are available within 24 h after blood has been taken, do not require carefully prepared biological substrates (such as the brain homogenates), and use standard enzyme-linked immunosorbent assay equipment and so could therefore be the basis of a rapid relatively high-throughput assay to determine the infectivity status of a flock.

The mean absorbance value for the VRQ/VRQ sheep with scrapie was slightly higher than the other groups (Fig. 1), suggesting variation in the amount of PrP^{sc} circulating in the blood. Such variation may be a result of one or a combination of genotype, inoculation route, dose, and strain. The origin of blood-borne prion is unclear but could potentially be a result of cells recirculating from the lymphoid tissues. The amount of PrP^{sc} accumulated in the lymphoid tissue is known to be influenced by PrP genotype (30). However, genotype is unlikely to be the sole reason for the differences observed here since high levels of accumulation in the lymphoreticular system (LRS) do not always result in detection of PrP^{sc} in blood (see below).

The detection rate observed in the present study (~56% for all animals tested) is lower than that observed by Castilla et al. (5), who observed that the blood samples from 89% of hamsters terminally ill with experimental scrapie were positive using the highly sensitive PMCA method. In addition, we have previously detected PrP^{sc} in the blood from sheep with scrapie using PMCA with a detection rate of 100% in 10 clinically affected VRQ/VRQ sheep (29), and this is consistent with a 100% transmission rate of infectivity from the blood of clinically affected sheep (20). PMCA can amplify very low levels of PrP^{sc}. Clearly, the plate assay described here does not match

that level of sensitivity, and lack of detection may be related to very low levels of PrP^{sc} rather than its complete absence.

Previous studies have shown that not all infectivity is associated with cells and indicate that plasma may also be infectious (13, 14). We have been unable to detect PrP^{sc} in plasma using our method, most probably because we are unable to concentrate the plasma sufficiently to apply to a single well without significant deleterious effects on the assay, as determined using plasma spiked with infected tissues (data not shown).

Detection of PrP^{sc} in the blood of sheep with preclinical scrapie. To investigate further the presence of PrP^{sc} in blood, we analyzed samples collected from sheep naturally exposed to scrapie during the preclinical phase of the disease. In sheep aged between 3 and 12 months, the levels of PrP^{sc} were significantly above background in 4 of 12 sheep (Fig. 2 and Fig. 3A and C). These data demonstrate that infected PBMC are circulating in the blood from 5 to 6 months of age, at least 12 months prior to clinical disease. PrP^{sc} accumulates in the LRS during the early phase of the disease (1, 31), and this suggests that lymphoid tissue may act as a reservoir for prions in the blood during this time. However, the observation that detection was intermittent suggests that infected PBMC may be transiently circulating through the blood.

At later time points, PrP^{sc} was detected in PBMC from 9 of 11 sheep analyzed (Fig. 2 and 3). Detection of PrP^{sc} was most consistent from the blood of the five VRQ/VRQ sheep aged between 14 months of age and clinical endpoint (age at death, 17 to 22 months). Two of the five had detectable PrP^{sc} at all sampling points up to clinical disease and death (Fig. 3B).

FIG. 3. PrP^{Sc} detection in blood during the preclinical phase in sheep naturally exposed to scrapie. PBMC were sampled and tested monthly from six VRQ/VRQ (V/V) sheep between 3 and 12 months of age (A), five V/V sheep between 14 months of age and the clinical endpoint (B), six ARQ/VRQ (A/V) sheep between 3 and 12 months of age (C), and five A/V sheep between 14 months of age and the clinical endpoint (Sh18 to Sh21) or 14 to 30 months of age (Sh22) and one ARR/VRQ sheep (Sh23) between 14 and 30 months of age inclusive (D). The values in the graph key represent months of age. All of the V/V sheep in panel B succumbed to clinical disease at between 18 and 24 months of age. In four of the six A/V sheep (five ARQ/VRQ and one ARR/VRQ) in panel D PrP^{Sc} was demonstrated in RAMALT biopsy specimens at 26 months (see Fig. 2 and Table 1). Mean absorbance values (standard error, <4.5%) shown have been normalized by subtracting the means of the 10 negative control blood samples for each assay. The dashed green lines represent the value of three SDs above the mean of the negative controls. Any value above the line is considered positive. The values derived from blood at postmortem from those sheep that succumbed to clinical disease ($n = 9$) are included in Fig. 1.

Three of the five had detectable PrP^{Sc} at at least one but not all time points.

In blood from the ARQ/VRQ sheep PrP^{Sc} was detected in four of five animals sampled from 14 months of age (Fig. 3, Sh18 to Sh21). Those four positive sheep all subsequently succumbed to clinical disease (the range of age at death was 28 to 35 months). The remaining negative ARQ/VRQ sheep in this group is still alive and not showing clinical signs at the time of writing. Similarly, PrP^{Sc} was not detected in the one ARR/VRQ sheep (Sh23) sampled during this period. Interestingly, PrP^{Sc} detection in PBMC from the ARQ/VRQ group was observed later than in the VRQ/VRQ sheep, for example, at 16 months of age four of five VRQ/VRQ sheep were PBMC positive compared to one of five positive sheep in the ARQ/VRQ group. The longer incubation periods of the ARQ/VRQ

sheep suggest that these observations were linked and may relate to the delayed LRS involvement in these sheep.

At 26 months of age the six sheep in the ARX/VRQ (ARR/VRQ and ARQ/VRQ) group were not showing clinical signs of disease, so RAMALT biopsy was performed to determine whether they were preclinically infected (11). PrP^{Sc} was detected in the RAMALT from all four sheep that had had at least one positive blood result (Table 1 and Fig. 3, Sh18 to Sh21). Conversely, the two sheep with consistently negative blood results during this period were also RAMALT negative (Sh22 and Sh23, Table 1 and Fig. 3), suggesting that they may not be infected with the scrapie agent. Alternatively, the sheep could be infected, but in the absence of PrP^{Sc} accumulation in RAMALT. In support of the latter view, it is highly likely that Sh22 will eventually develop disease since all ARQ/VRQ an-

TABLE 1. PrP^{Sc} detection by IHC in RAMALT biopsy specimens of ARQ/VRQ and ARR/VRQ sheep exposed to scrapie agent^a

Animal	RAMALT diagnosis	% Follicles PrP ^{Sc} positive
Sh18	Positive	80
Sh19	Positive	86
Sh20	Positive	88
Sh21	Positive	97
Sh22	Negative	0
Sh23	Negative	0

^a Sheep were 26 months of age at time of biopsy. PrP^{Sc} was detected by IHC using PrP C-terminal antibody, R145.

imals that have been born into the flock and survived past 26 months of age have gone on to develop disease with a median incubation period of 31 months (range, 4 to 48 months) (J. Moore, unpublished data).

These data indicate that the presence of PrP^{Sc} in PBMC of preclinical scrapie agent-infected sheep may depend on multiple factors; the PrP genotype, the involvement of the LRS, the length of the incubation period, and the stage of the disease when blood is sampled. PrP^{Sc} sequentially accumulates first in gut-associated lymphoid tissue and draining lymph nodes in orally and naturally infected sheep (1, 27, 31). Sheep homozygous for VRQ have the earliest and most widespread LRS involvement: PrP^{Sc} can be detected in Peyer's patches and mesenteric lymph nodes as early as 2 to 4 months of age in an infected flock (1, 31), followed by a rapid and progressive increase in accumulation throughout the LRS. Furthermore, the extent and rate of accumulation of PrP^{Sc} in the LRS is dependent on the genotype of the sheep (8, 30). In the present study the detection of PrP^{Sc} in PBMC from sheep as early as 5 months is consistent with the involvement of the blood in the dissemination of the agent within the LRS. However, we observed that (i) not all sheep likely to have PrP^{Sc} in the lymphoid tissue were PBMC positive in the early stages of the disease and (ii) the presence of positive PBMC is transient even at the later stages of the disease in some animals, suggesting that the presence of prions in LRS and blood are not necessarily coincident. However, that VRQ/VRQ sheep are more likely to have positive PBMC earlier than ARQ/VRQ sheep is consistent with the findings that the propagation of PrP^{Sc} in the LRS is slower and less consistent in ARQ/VRQ sheep (8) and indeed often absent in ARR/VRQ sheep (30).

Transmission studies in sheep showed that infectivity in the blood was present during the preclinical phase of the disease (17, 18, 20), although transmission was more successful when donor sheep were more than half way through the incubation period. Blood collected in the first half of the incubation period produced a lower rate of transmission. The data from this transfusion study are consistent with our observations that PrP^{Sc} is more readily detected in the blood in the second half of the incubation period.

Preclinical detection of prions in the blood using PMCA has been reported after experimental intraperitoneal inoculation of hamsters with scrapie (28). However, a biphasic distribution of prions was observed in that study: the first peak during the preclinical period, at approximately one-third of the incubation period, and the second peak at the clinical endpoint. The authors' explanation for this phenomenon is that the first peak

coincides with the exponential replication of prions in lymphoid tissue and the second peak coincides with the leakage of cerebral proteins into the blood following disease-related damage in the central nervous system. It is not apparent from our study that there is a biphasic prion distribution in blood from naturally infected sheep (Fig. 3). However, several explanations could account for these differences, such as the route of inoculation, host variability, and the length of the preclinical phase.

We observed variability in the detection of PrP^{Sc} in PBMC in sequential monthly samples from individual sheep. It is unclear why the amounts of PrP^{Sc} associated with blood leukocytes should vary between sampling points. One explanation is that the trafficking of infected cells from the point of infection is influenced by factors such as exposure to other pathogens or perhaps there is an inherent instability of the association of PrP^{Sc} with blood cells that is affected during the *in vitro* processing of the blood. The latter is currently under investigation. In a commercial or surveillance setting the variable presence of PrP^{Sc} in blood would reduce the reliability of a blood test performed at a single time point. This could be overcome by sequential blood sampling over a period of months.

Estimation of the proportion of infected cells in the blood. In order to estimate the proportion of possible infected lymphoid cells in blood, we compared the absorbance values obtained from serially diluted cells dissociated from the mesenteric lymph nodes. Serial dilutions of dissociated lymph node cells (DLN) were dispensed into the wells of the assay plate, and the minimum cell number required to produce a positive optical density value for a sheep with the VRQ/VRQ or ARQ/VRQ genotype was calculated as ~16,000 cells (Fig. 4). A previous study reported (15) that the number of DLN with detectable PrP^{Sc} labeling by the immunohistochemistry (IHC) method in a lymph node is 2%. This is an approximation determined by counting very limited numbers of cells. We have also assumed that the sensitivity of the IHC method and the plate assay are equal and that the levels of accumulation of PrP^{Sc} associated with individual PBMC are similar to cells of the lymph node. With these caveats acknowledged, the number of PrP^{Sc}-positive DLN required per well to enable the detection of PrP^{Sc} in the immunoassay was estimated and found to be ≥320 PrP^{Sc}-positive cells per well. Thus, we have approximated the range of possible infected cells in blood from <320 positive cells (per 10⁷ PBMC) to 10,000 positive cells (per 10⁷ PMBC). Therefore, the proportion of positive cells in the blood may range from less than 1/20,000 (<0.005%) to 1/1000 (0.1%) PBMC; about 20- to 500-fold less than in the lymph nodes. These calculations are based on the assumptions that blood-borne prion-infected cells originate in the lymph nodes and that they bear similar quantities of prion protein to the DLN. However, if the amount of PrP^{Sc} associated with each blood-borne cell is lower than that observed in each cell in a lymph node, then the proportion of infected cells in blood could be higher.

Conclusion. These results indicate that the circulating PBMC of sheep with TSE harbor PrP^{Sc} in sufficient quantities to be detected by a highly sensitive ligand-based rapid immunoassay. Preclinical detection indicates that screening of blood in the absence of clinical signs is possible. This is the first study to demonstrate PrP^{Sc} in blood in the absence of amplification

FIG. 4. Titration of DLN from scrapie-positive sheep (closed symbols) at the clinical endpoint from VRQ/VRQ (circles) and ARQ/VRQ (squares) sheep. Lymph nodes from three unexposed sheep (open symbols) were included as controls. The minimum number of DLN required to give an absorbance value greater than 0.056 for these individual sheep is ~16,000 cells (inset, dashed line). The maximum value observed for PBMC was 0.35 (main graph, dashed line) and equates to a maximum of ~540,000 DLN.

or bioassay and provides the basis of a high-throughput screening assay. Currently, the most laborious step is the isolation of the cells from blood, which must be freshly sampled from animals. We are evaluating methods to reduce the time required for this step by enriching for the positive cell fraction directly from whole blood, which would eliminate the need for gradient centrifugation and facilitate automation. Furthermore, PrP^{Sc} was not detected in the blood of every infected sheep. Host factors controlling the recirculation and clearance of infected blood cells may be involved. Further study is required to fully understand the pathogenesis events that control blood involvement during disease. Nevertheless, this assay could be adapted for human PrP, and early diagnosis of infected individuals could have a beneficial impact on the future management of the disease.

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L.H., J.H., and J.C.E. performed blood separations, biochemical assays, and data analysis. S.J.B. and H.S. managed the projects supplying the sheep blood. L.A.T. proposed the study, analyzed the data and wrote the manuscript. S.J.E. managed the projects. S.J.M. performed the IHC examination and edited the manuscript. S.L., L.E., and V.L. helped develop the assay and provided intellectual input and helpful discussions.

The authors declare a competing financial interest (S.L., L.E., and V.L.).

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