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Raman Analysis of Prion Protein in Blood Cell Membranes from Naturally Affected Scrapie Sheep

Brief Communication

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Summary

At present, there are no efficient antemortem diagnostic tests for transmissible spongiform encephalopathys (TSEs), particularly in blood. New strains of prion PrPSc, which causes TSEs, are currently appearing, and researchers remain concerned that if prion variants continue to emerge, some of them may escape detection by existing immunoassay tests. Because a common structural feature of PrPSc strains is their high content of β sheets, Raman spectroscopy has proven to be a suitable technique to analyze a key membranous fraction of blood containing PrPSc. In this fraction, a significant increase in β sheets has been correlated with the worsening of this TSE in naturally scrapie-infected animals in comparison with healthy controls. Since sensitivity and specificity were found to be 100% for each, this test may lead to a new and alternative diagnosis for prion diseases.

Introduction

Transmissible spongiform encephalopathies (TSEs), such as scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle, or variant Creutzfeldt-Jakob disease (VCJD) in humans, are a group of deadly neurodegenerative diseases caused by a family of unusual pathogenic prion proteins. Conformational changes of the cellular prion protein (PrP) play a key role in the pathogenesis of TSEs or prion diseases and are widely considered to be fundamentaly involved in their etiology. A major goal for both medical and veterinary research is the development of a rapid, in vivo, highly sensitive test that could identify a TSE during the preclinical period of the disease [1, 2]. Scrapie is a naturally occurring prion disease, widely spread over the world. Human blood infectivity studies have yielded conflicting results: infectivity has rarely been detected in naturally acquired disease, but in experimentally infected rodents it has been found at very low levels during both the incubation period and clinical phase of the disease [3, 4]. Concerning scrapie, one of the best models for studying neurodegenerative diseases that lead to a fatal end involving spongiosis and accumulation of proteinase-resistant prion protein (PrPSc) in amyloid plaques [5], it has been recently shown that blood from naturally infected scrapie animals is infectious [6], although fluid infectivity in experimentally infected sheep had already been previously demonstrated [7]. These observations have prompted the use of various tests to detect in blood the presence of proteinase-resistant prion protein PrPSc, which appears to be an accurate marker of infectivity [4, 8].

PrPSc has been reported to be detected by a sensitive immunocompetitive capillary electrophoresis (ICCE) assay in clinical scrapie of sheep with naturally acquired disease [9]. However, this study was limited by a low number of samples, and the technique requires further validation. In addition, this method has been reported to be unsuitable for use as a screening test in human TSEs, because the test is unable to distinguish between blood extracts from healthy subjects and patients affected with various forms of TSEs [3]. In addition, this test, among other difficulties, is time consuming. Other investigations using methods of immunohistochemistry failed to demonstrate PrPSc in peripheral blood leucocytes of scrapie-infected sheep [10]. Regarding the tests for PrPSc detection developed up to date, they have been widely shown to have high sensitivity in the central nervous system and peripheral lymphoid tissue, but seem not to be able to detect it in blood [11, 12]. Moreover, routine tests reveal unknown strains of prions, and therefore researchers remain concerned that if prion variants continue to emerge as they have, some of them may escape detection by existing tests [13]. Consequently, a new test that does not involve antibodies is desirable. A method based on Fourier-transform infrared spectroscopy has been reported to provide a reliable blood serum test for scrapie and other TSEs [14]. However, the spectral differences shown through this test do not involve detection of PrPSc, which is known to be the ethiological agent of TSEs. Moreover, with respect to the pathological alterations observed in the sera of the scrapie-infected hamsters studied in this infrared work [14], it needs to be addressed whether and to what extent they are specific for scrapie and other TSEs.

Raman spectroscopy is a laser-based type of spectroscopy that presents information on the chemical composition and structure of biological molecules and systems. Although the Raman spectra of cells may include spectra of various molecular compounds present in the cells, in this particular research we have studied membrane-enriched fractions of blood cells where prion proteins are located in cases of naturally scrapie-infected sheep. The analytical selectivity arises from the fact that the predominant β structure of infective protein in these fractions is clearly visible in the Raman amide I spectral region. In this regard, it should be noted that the key to the prion infectivity is the transition of the noninfective isoform PrPC to the infective PrPSc, which is rich in

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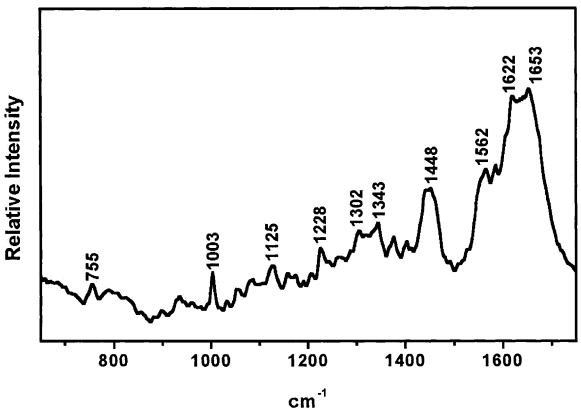


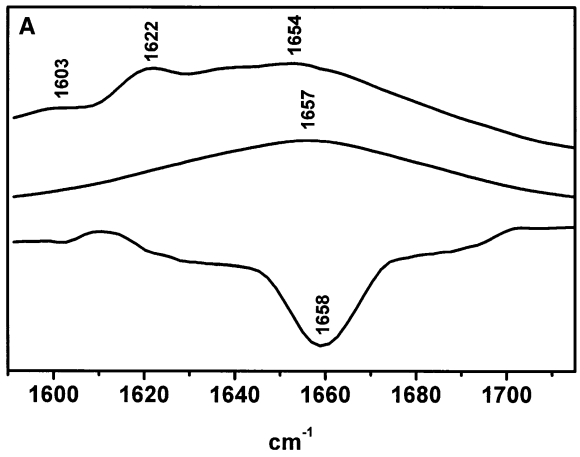
Figure 1. Raman Spectrum of a Membranous Blood Pellet from a Scrapie-Infected Sheep

β sheets [15, 16]. In this connection, we have found significant differences in β-structure concentrations in healthy and infected animals, which can be used to classify sample spectra as a diagnostic tool for identification of even preclinical stages of scrapie disease. It should be noted that, since β sheets as the predominant structure is a common feature in all PrPSc, Raman spectroscopy solves the inconvenience of unknown prion strains escaping detection by current immunological tests.

Results and Discussion

Figure 1 includes the Raman spectrum of a blood membrane pellet, showing that the predominant character of this fraction is proteic, as reflected by the amide I band located at 1653 cm^{-1}, by the amide III bands near 1300, 1250, and 1240 cm^{-1}, and by some protein side chain bands generated by tryptophan (1562, 1343, and 755 cm^{-1}) and phenylalanine (1003 cm^{-1}). Rests of hemoglobin are visible through the bands located near 1620, 1550, 1377, 1230, and 1125 cm^{-1} [19]. Phospholipid components of these pellets are also present, as shown by their strongest bands near 1450, 1300, 1090, and 1060 cm^{-1} [20].

Figure 2 shows the Raman spectra of a membrane pellet from blood control. The top spectrum includes a double-bond stretching band at 1622 cm^{-1} generated by the hemin prosthetic group of hemoglobin, which is, in minor amounts, present in the pellet. It is notable that Raman intensity of the above hemin group rests is relatively strong at this frequency compared with the intensity from the protein part in the amide I band. However, since hemin does not have strong absorption bands in the 800-1200 cm^{-1} region [19], our 1064 nm excited spectra are nonresonant. In all probability, the highly polarizable nature of hemin prosthetic group gives strong Raman scattering intensities. Before calculating the percentage of β structure, the bands overlapping with the amide I spectral profile were subtracted from the original spectra according to well-known methods [18]. The resulting spectrum (Figure 2A, middle and bottom) exhibits a band centered near 1657 cm^{-1}. This



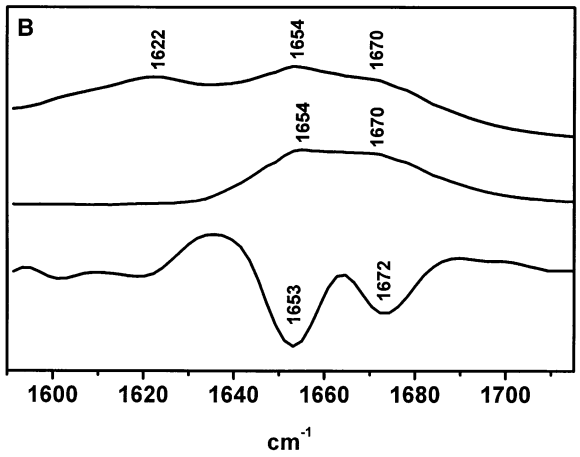


Figure 2. Raman Spectra of a Blood Membrane Fraction

(A) shows spectra from a healthy control animal, and (B) shows spectra from a scrapie-infected animal. Original spectrum, top; amide I band spectrum, middle; subsequent second derivative spectrum, bottom.

spectrum provides spectral information of all proteins present in the membranous blood fraction obtained in this work. The potential presence of nucleic acids can be neglected because of the absence of phosphoester characteristic bands of these biomolecules in the 900-800 and 1300-1000 cm^{-1} ranges and by the absence of nucleobase bands in the 1680-1710 cm^{-1} region. Phospholipids, which have no band interfering in the amide I region [20], should not be previously removed from this fraction for posterior Raman analysis. Due to overlapping of the amide I bands of proteins [20], spectral profiles with signals attributable to specific protein constituents on a molecular basis are not possible at present. However, previous knowledge of the probable presence of PrPSc, which have β sheets as the predominant secondary structure, permits assignments of β sheet band intensity to variations in PrPSc concentration. In healthy controls, no signal attributable to β sheets is clearly visible in its 1665-1680 cm^{-1} characteristic range (Figure 2A) [15, 16, 20]. In contrast, Figure 2B shows Raman spectra measured from a scrapie- infected sam-

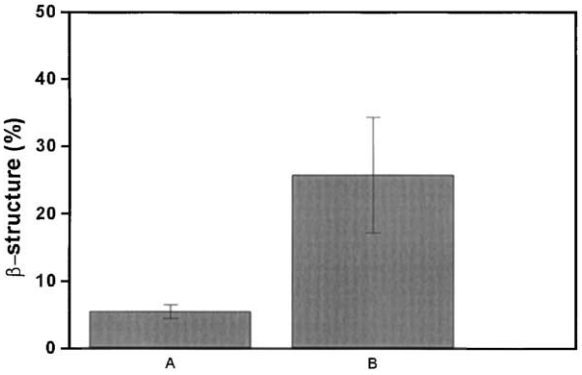


Figure 3. Bar Diagram for the Means of β-Sheet Percentages Measured for Healthy Control and Scrapie-Infected Samples from Genetically Selected Animals

A indicates healthy control and B indicates scrapie-infected samples. Error bars correspond to standard deviation. The β sheet contents from the series of scrapie samples (31 sheep) were found to be significantly different ($p < 0.05$) from those of the healthy negative controls (150 sheep).

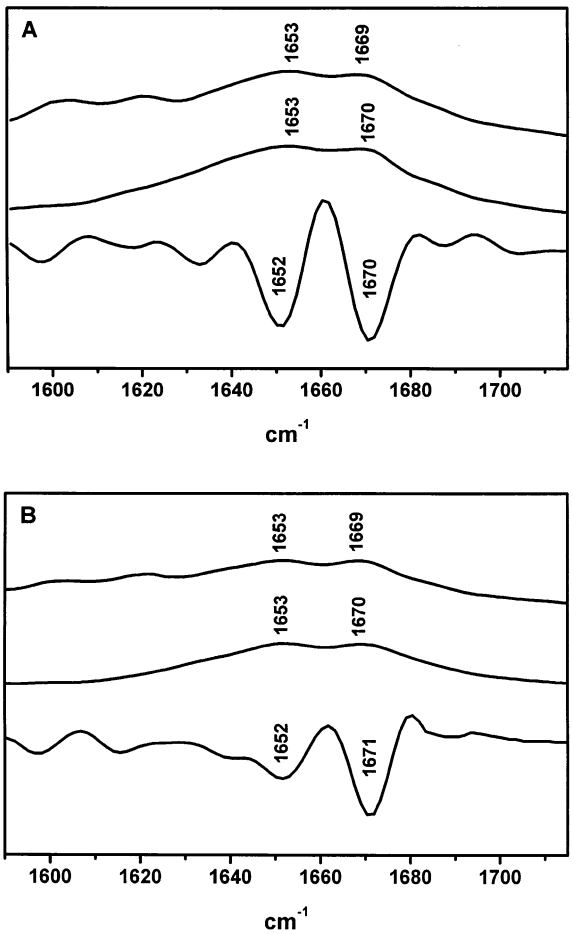


Figure 4. Raman Spectra of a Blood Membrane Fraction

(A) shows spectra from a scrapie-infected animal, and (B) shows spectra of the same animal one month later. Original spectrum, top; amide I band spectrum, middle; subsequent second derivative spectrum, bottom.

ple; a band near 1670 cm^{-1} is apparent. According to the histograms correlating protein structure and amide I frequencies [20], a protein amide I frequency falling at 1670 cm^{-1} can be unambiguously considered indicative of β structure. Furthermore, amyloid protein β sheets have been reported to generate amide I bands near 1668 cm^{-1} [21].

On the basis of the measured spectroscopic signals and amounts of the obtained membrane-rich fractions, this technique can detect up to about 10 μg/ml prion concentration present in bulk blood, which is of the same order of magnitude as the concentrations detected by ultraviolet and fluorescence spectroscopies [4]. On the other hand, the percentages of β sheets were obtained from the original spectra but not from second derivative spectra. Although the original ones seem to be relatively broad in the amide I band region, it is well known that slight variations in the frequency of the amide I intensity maximum and bandwidth can result in clearly different percentages in secondary structures [18]. The amide I band in the infrared spectrum can also be used to detect prion proteins, using either the transmission or ATR option. However, unlike the Raman technique, the very strong water absorption in this infrared region cannot be reliably subtracted. Consequently, the best infrared solution is to use deuterated samples (a time-consuming task) to remove the δH_2O infrared band from the amide I spectral range. Hence, the comparison of Raman and infrared techniques at this level makes Raman spectroscopy a more direct technique than infrared spectroscopy for the study of these samples. Moreover, in the infrared study of dried films from blood serum that was carried out by other authors [14], the amide I band region was neglected, and therefore prion proteins as causal agents of TSEs were not detected in that investigation.

A question arises as to whether the above β sheet structure corresponds unambiguously to the infective prion protein. A positive answer is supported by the following facts. First, there seems to be a clear correspondence between percentages of β sheet secondary structure and absence or presence of scrapie disease (Figure 3). The bar graphs in Figure 3 show the β sheet contents from the series of scrapie samples (31 sheep registered in our flock so far), which were found to be significantly different ($p < 0.05$) from the healthy negative controls (150 sheep). All of the β sheet percentages falling in the range over 10% (Figure 3) correspond here to scrapie-infected blood samples, all of them showing the characteristic β sheet shoulder near 1670 cm^{-1}. The β sheet contents are obviously more scattered in the infected samples than in the healthy ones (Figure 3), due to the different disease stages in scrapie progression. Second, the assignment of the 1670 cm^{-1} band to PrPSc is consistent with the increase in β sheets found here over the scrapie disease course (Figure 4). Moreover, from the literature it is known that only some neurodegenerative diseases involve amyloid deposition generated by proteins with characteristic high β sheet structure percentages [21]. Third, the spectroscopic results here have been found to be in agreement with the inmunohistochemical analysis of the medulla oblongata re-

gion (obex) of sheep brain. Furthermore, it is not surprising that the membranous fraction obtained here through osmotic shock and appropriate centrifugation contains PrPSc, since it is reported that these prion molecules are mainly anchored on cell membranes, endosomes, and the Golgi compartments. [8, 22, 23]. Thus, PrPC is associated with the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor [24], and several experimental observations support the suggestion that an interaction of PrP with the lipid membrane can be involved in the process of conversion of PrPC into PrPSc. Among them is the fact that PrPC is easily cleaved upon treatment with the enzyme phosphatidylinositol-specific phospholipase C and removed from the membrane surface. However, upon similar treatment, PrPSc is retained on the membrane surface [25, 26], which strongly supports the idea that an altered membrane association of PrP may be an important factor in the mechanism of prion diseases [27]. On the basis of the above data and the fact of the involvement of the lymphoreticular system in scrapie pathogenesis [6, 28-30], finding PrPSc in the membranous fraction of blood cells can be reasonably expected, as shown in this work. Thus, as to the specific blood cells where prion protein can be identified, white blood cells of the immune system, particularly B lymphocytes, have been reported [8, 30]. In this connection, we have obtained results indicating that PrPSc is located in blood fraction of lymphocytes.

Sensitivity, as an indicator of capability to detect positive scrapie cases, was found to be 100%. On the other hand, according to the OIE (Office International des Epizooties, regulations 1248/2001, Paris, France), specificity defines the probability of a test to recognize truly negative samples as negative. Taking into account that all of the negative samples studied here (checked postmortem by inmunohistochemistry methods of brain) show β sheet percentages that are clearly separated from those of the scrapie samples, it is reasonable to say that specificity of this Raman test for the samples studied has been 100%. In the group of 150 healthy animals that have been studied here, the β sheet percentages were found to be between 4% and 7%. By contrast, in the 31 animals appearing infected so far, for the last two years, said percentage ranged from 10% to 40%, depending on the disease severity. Therefore, the cut-off is located between 7% and 10% of β sheet percentages, with no significant outliers. The number of sheet cases studied here is statistically relevant, because from a large number of animals, 150 sheep were genetically selected for their susceptibility to scrapie disease. The susceptibility of our flock was found to be 5000 times greater than in the absence of genetic selection. There are very few flocks with this genetic characteristic. One of them is ours, and this has proven to be useful and applicable for investigation of naturally acquired TSEs.

Significance

Our spectroscopic results reveal that Raman analysis of a key membrane fraction isolated from blood can provide a new rapid in vivo test for diagnosis of scrapie. Because scrapie can cause infectivity in cattle, it is expected that this procedure may be applicable to other TSEs whose study is in progress. At present, we have not detected infective prion proteins either in concentrated blood plasma or serum, which can be attributable to the fact that prion proteins are membrane-anchored glycoproteins. Moreover, this procedure involves the use of blood, which is easily accessible and may therefore serve as an appropriate test material. Unlike other methods that use dried films of test materials to enhace the spectroscopic signal/noise ratio [14], here we have used aqueous samples in order to avoid protein aggregation or artifact results. In addition, this method is rapid, reagent free, and nondestructive and could also be used as an alternative tool to elucidate often ambiguous results obtained by other immunological validated tests [31], particularly when new prion variants could be responsible for the TSEs and therefore present some difficulty in being distinguished [13]. Finally, the screening of living flocks of sheep, goats, and cattle would be valuable to control and eradicate the TSEs in these animals and to prevent human-to-human transmission via blood or blood product transfusion, because there is not full confidence in the current methods for diagnosis of TSEs [32]. Therefore, the application of our findings would solve a problem that has great scientific, economic, and public health importance. Consequently, this technique shows considerable promise as an accurate antemortem diagnostic test for TSEs, and we think it will be of interest for a wide rage of scientists in this field and for other important groups of neurological diseases.

Experimental Procedures

Livestock and Blood Sampling

Blood samples were obtained from a special flock consisting of 150 sheep, over four years old, genetically selected for their susceptibility to contract and develop scrapie disease. This scrapie-sensitive group was selected from a large number of animals. Additionally, 150 healthy sheep corresponding to flocks of the above-mentioned age were used as negative control animals. All of them were adult female sheep with high-susceptibility genotype (ARQ/ARQ, ARQ/ARH, or ARR/ARQ).

Necropsy was performed inmmediately after natural death or slaughtering the animal (by intravenous injection of sodium pentobarbital and exsanguination). The brain was removed from each sheep for scrapie diagnosis confirmation. Histopathology and immunohistochemistry procedures previously described [17] were chosen for confirmation of all spectroscopic results. Briefly, monoclonal L42 antibody (R-Biopharm, Germany; 1:500) after formic acid, hydrated autoclaving, and proteinase K digestion treatments, EnVision, and diaminobenzidine (DAKO, Denmark) were all used for PrPSc detection. Moreover, PrPSc presence/absence was also assessed in other tissues from the lymphoreticular system in order to dismiss possible preclinical cases. The results obtained through these procedures were continuously compared with the evolution of clinical symptoms of the flock.

Blood samples were collected before culling in all cases and placed into 10 ml sterile tubes containing 1.4 mM EDTA to avoid blood coagulation. Blood sample collection was carried out to permit the isolation of a key blood membrane fraction where prion proteins are concentrated. Steps consisting of centrifugation and osmotic shock (as described below) for getting a prion-rich pellet were essential. Specific staining and further morphological examination by light microscopy was applied for cellular component identifi-

cation within the obtained fraction. We have also carried out assays to detect the presence of fibrilar forms of the scrapie prion in our blood membrane samples, but without conclusive results. This is due, among other problems, to the lability of these samples and the fact that these assays are conceived preferently for fixed tissues. It would be necessary to develop a new procedure specifically designed to this type of labile sample.

Preparation of the Membrane-Rich Fraction from Blood Cells

About 6 ml of blood was centrifugated at 4300 $\times$ g for 30 min at 4°C. The resulting pellet was then completely resuspended with saline isotonic aqueous solution. The suspension was then centrifuged at the above conditions, and the resulting pellet was subjected to osmotic shock with up to 40 ml of water (Milli-Q, Millipore). The use of water here is supported by its dispersing ability relative to buffers. After the suspension became homogeneous, it was centrifuged at 12,000 $\times$ g for 30 min at 4°C, and the supernatant was completely decanted, which led to a small membranous pellet used for Raman analysis. This general procedure can be shortened through direct osmotic shocks of blood in water and subsequent centrifugation and pellet analysis, but in this way Raman sensitivity for prion detection is reduced to about 10%.

Raman Spectroscopy

Raman spectra were obtained using a Bruker RFS 100/S FT spectrometer. Samples of membranous pellets for Raman analysis were placed inside NMR tubes of 5 mm inner diameter and were thermostatized at 20 $\pm$ 0.5°C. The volumes of the pellets were about 200 μl and had a protein concentration around 40%. A Nd:YAG laser operating at a wavelength of 1064 nm with about 300 mW of power was used as the excitation source. The scattered radiation was collected at 180° to the incident beam. Typical spectra were recorded at 4 cm^{-1} resolution, and the frequency-dependent scattering that occurs in the Raman spectra recorded with this spectrometer was corrected by multiplying point by point with $(\nu_{laser}/\nu)^4$. Frequencies cited are accurate to $\pm$ 0.5 cm^{-1} as checked through frequency calibration using an indene standard. Five hundred scans were accumulated and averaged to produce spectra with a good signal-to-noise ratio. For spectral measurements of the samples, they were placed into a thermostated device at about 20°C. Signals obtained were fed to a microcomputer for storage, display, plotting, and processing, and the manipulation and evaluation of the spectra were carried out using the Opus2 software supplied by Bruker. The Raman method that has been often used to determine protein secondary structure involves the use of parametric equations derived from a wide set of model proteins having various known secondary structures [18]. The β sheet percentages can be determined by using in these equations protein spectral parameters of the amide I region. In order to obtain pure amide I spectral profiles for determination of protein secondary structure, the heme band near 1620 cm^{-1} as well as the aromatic one near 1605 cm^{-1} were subtracted. With this aim, previous curve fitting of the original spectrum to a sum of Gaussian band components was carried out. The percentages of β sheet secondary structure were determined according to literature methods [18], based on analysis of amide I band spectral profiles.

Statistic Analysis

This was done using unpaired Student's t tests comparing the two series of spectroscopic results corresponding to healthy and scrapie infected animals. $p < 0.05$ was considered statistically significant.

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References

1. Prusiner, S.B. (2001). Neurodegenerative diseases and prions. N. Engl. J. Med. *344*, 1516–1526.
2. Aguzzi, A., Montrasio, F., and Kaeser, P.S. (2001). Prions: health scare and biological challenge. Nat. Rev. Mol. Cell. Biol. *2*, 118–126.
3. Cervenakova, L., Brown, P., Soukharev, S., Yakovieva, O., Diringer, H., Saenko, E.L., and Drohan, W.N. (2003). Failure of immunocompetitive capillary electrophoresis assay to detect disease-specific prion protein in buffy coat from humans and chimpanzees with Creutzfeldt-Jakob disease. Electrophoresis *24*, 853–859.
4. Brown, P., Cervenakova, L., and Diringer, H. (2001). Blood infectivity and the prospects for a diagnostic screening test in Creutzfeldt-Jakob disease. J. Lab. Clin. Med. *137*, 5–13.
5. Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W., and Glabe, C.G. (2003). Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science *300*, 486–489.
6. Hunter, N., Foster, J., Chong, A., McCutcheon, S., Parnham, D., Eaton, S., MacKenzie, C., and Houston, F. (2002). Transmission of prion diseases by blood transfusion. J. Gen. Virol. *83*, 2897–2905.
7. Houston, F., Foster, J.D., Chong, A., Hunter, N., and Bostock, C.J. (2000). Transmission of BSE by blood transfusion in sheep. Lancet *356*, 999–1000.
8. Herrmann, L.M., Davis, W.D., Knowles, D.P., Wardrop, K.J., Sy, M.-S., Gambetti, P., and O'Rourke, K.I. (2001). Cellular prion protein is expressed on peripheral blood mononuclear cells but no platelets of normal and scrapie-infected sheep. Haematologica *86*, 146–153.
9. Schmerr, M.J., Jenny, A.L., Bulgin, M.S., Miller, J.M., Hamir, A.N., Cutlip, R.C., and 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.
10. Herrmann, L.M., Baszler, T.V., Knowles, D.P., and Cheevers, W.P. (2002). PrPSc 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.
11. Deslys, J.P. (2003). Prions and risks for blood transfusion: the situation in 2003. Transfus. Clin. Biol. *10*, 113–125.
12. Schreuder, B.E.C., van Keulen, L.J.M., Vromans, M.E.W., Langeveld, J.P.M., and Smits, M.A. (1996). Preclinical test for prion diseases. Nature *381*, 563.
13. Willerroider, M. (2003). Routine tests reveal unknown strains of BSE prions. Nature *425*, 648–649.
14. Lasch, P., Schmitt, J., Beekes, M., Udelhoven, T., Eiden, M., Fabian, H., Petrich, W., and Naumann, D. (2003). Antemortem identification of bovine spongiform encephalopathy from serum using infrared spectroscopy. Anal. Chem. *75*, 6673–6678.
15. Caughey, B., Raymond, G.J., and Bessen, R.A. (1998). Strain-dependent differences in β-sheet conformations of abnormal prion protein. J. Biol. Chem. *273*, 32230–32235.
16. Caughey, B.W., Dong, A., Bhat, K.S., Ernst, D., Hayes, S.F., and Caughey, W.S. (1991). Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy. Biochemistry *30*, 7672–7680.
17. Badiola, J.J., Monleón, E., Monzón, M., Acín, C., Luján, L.L., Fernández, D., Simmons, M., and Vargas, A. (2002). Description of the first cases of BSE in Spain. Vet. Rec. *151*, 509–510.
18. Williams, R.W. (1983). Estimation of protein secondary structure from the laser Raman amide I spectrum. J. Mol. Biol. *166*, 581–603.
19. Ozaki, Y., Mizuno, A., Sato, H., Kawauchi, K., and Muraishi, S. (1992). Biomedical application of near-infrared Fourier transform Raman spectroscopy. Part I: the 1064 nm excited Raman spectra of blood and met hemoglobin. Appl. Spectrosc. *46*, 533–536.
20. Tu, A.T. (1982). Raman Spectroscopy in Biology. Principles and Applications. (New York: John Wiley & Sons), pp. 74.

21. Antzutkin, O.N., Leapman, R.D., Balbach, J.J., and Tycko, R. (2002). Supramolecular structural constraints on Alzheimer's β-amyloid fibrils from electron microscopy and solid-state nuclear magnetic resonance. Biochemistry *41*, 15436–15450.
22. Caughey, B., and Kocisko, D.A. (2003). A nucleic-acid accomplice? Nature *425*, 673–674.
23. Brown, L.R., and Harris, D.A. (2003). Copper and zinc cause delivery of the prion protein from the plasma membrane to a subset of early endosomes and the Golgi. J. Neurochem. *87*, 353–363.
24. Stahl, N., Borchelt, D.R., Hsiao, K., and Prusiner, S.B. (1987). Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell *51*, 229–240.
25. Stahl, N., Borchelt, D.R., and Prusiner, S.B. (1990). Differential release of cellular and scrapie prion proteins from cellular membranes by phosphatidylinositol-specific phospholipase C. Biochemistry *29*, 5405–5412.
26. Safar, J., Ceroni, M., Gajdusek, D.C., and Gibbs, C.J. (1991). Differences in the membrane interaction of scrapie amyloid precursor proteins in normal and scrapie- or Creutzfeldt-Jakob disease-infected brains. J. Infect. Dis. *163*, 488–494.
27. Sanghera, N., and Pinheiro, T.J.T. (2002). Binding of prion protein to lipid membranes and implications for prion conversion. J. Mol. Biol. *315*, 1241–1256.
28. Aucouturier, P., Carp, R.I., Carnaud, C., and Wisniewski, T. (2000). Prion diseases and the immune system. Clin. Immunol. *92*, 79–85.
29. Bons, N., Lehmann, S., Mestre-Frances, N., Dormont, D., and Brown, P. (2002). Brain and buffy coat transmission of bovine spongiform encephalopathy to the primate Microcebus murinus. Transfusion *42*, 513–516.
30. Klein, M.A., Frigg, R., Flechsig, E., Raeber, A.J., Kalinke, U., Bluethmann, H., Bootz, F., Suter, M., Zinkernagel, R.M., and Aguzzi, A. (1997). A crucial role for B cells in neuroinvasive scrapie. Nature *390*, 687–690.
31. Moynagh, J., and Schimmel, H. (1999). Tests for BSE evaluated. Nature *400*, 105.
32. Murphy, W.G. (2002). Disease transmission by blood products: past, present and future. Pathophysiol. Haemost. Thromb. Suppl. *32*, 1–4.