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Journal of Virological Methods 125 (2005) 187–193
Journal of Virological Methods
www.elsevier.com/locate/jviromet

An improved Western blot assay to assess the clearance of prion protein from plasma-derived therapeutic proteins

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Received 12 October 2004; received in revised form 17 January 2005; accepted 18 January 2005
Available online 24 February 2005

Abstract

Specific detection of the pathogenic prion protein, PrP^{Sc}, is essential for determining the prion clearance capacity of purification processes for therapeutic proteins. Use of a previously described indirect (two-antibody) Western blot assay sometimes resulted in the appearance of non-specific protein bands that interfered with the detection of small amounts of PrP^{Sc}-specific signal, limiting the amount of clearance that could be determined for steps so affected. It is shown that these non-specific signals are due to the interaction between immunoglobulin fragments in the sample and the secondary antibody used in the assay. To circumvent this problem, a direct Western blot assay using a prion-specific primary antibody conjugated to the reporter enzyme alkaline phosphatase was developed. Application of the direct Western blot assay resulted in a significant reduction of non-specific signal while retaining the detection sensitivity for PrP^{Sc}-specific signal. Therefore, the direct Western blot assay format is an improved tool for determining prion clearance capacity, particularly for immunoglobulin-rich samples.

Keywords: Prion protein; Plasma proteins; Immunoglobulin; TSE; Western blot assay; Non-specific signal

1. Introduction

The emergence of variant Creutzfeldt–Jacob Disease (vCJD) (Will et al., 1996), a human prion disease linked to bovine spongiform encephalopathy (BSE) (Bruce et al., 1997; Collinge et al., 1996; Hill et al., 1997; Scott et al., 1999), has prompted concerns of prion disease transmission through blood and blood products. Indeed, the potential for transmission by blood transfusions was demonstrated experimentally (Houston et al., 2000; Hunter et al., 2002) prior to the first apparent case of an actual transmission of vCJD by transfusion (Llewelyn et al., 2004). Although there are no reported cases, plasma-derived therapeutic proteins have also been implicated as potential sources for the transmission of prion diseases (Ironside and Head, 2003). However, unlike whole blood or blood component transfusions, plasma-derived therapeutic proteins are highly purified. Many of these purification processes have a significant capacity for prion removal, leading to an improved safety margin with regard to disease transmission (Burnouf and Radosevich, 2003; Cai et al., 2002; Foster et al., 2000; Gregori et al., 2004; Lee et al., 2001; Stenland et al., 2002; Tateishi et al., 2001; Trejo et al., 2003; Van Holten and Autenrieth, 2003).

An indirect Western blot assay to determine the prion removal capacity of various plasma protein purification processes was described previously (Lee et al., 2000). This assay was based on the selective detection of a buried structural element (3F4 epitope), which is unique to the pathogenic form of prion protein (PrP^{Sc}) (Peretz et al., 1997; Safar et al., 1998). In the normal cellular prion protein (PrP^{C}), this and many other structural elements are exposed to a solvent (Knaus et al., 2001; Matsunaga et al., 2001; Riek et al., 1997) and are therefore susceptible to proteolysis during sample manipulation, and no detectable signal is observed using the Western blot assay. However, PrP^{Sc} (e.g., strain 263 K hamster-adapted

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doi:10.1016/j.jviromet.2005.01.026

scrapie) undergoes only a partial proteolysis, resulting in the generation of a truncated proteinase-resistant core referred to as PrP^{RES}, which contains the buried 3F4 epitope. The protected 3F4 epitope of PrP^{RES} can then be exposed through denaturation and detected by the Western blot assay. A strong correlation between the detection of the 3F4 epitope on PrP^{RES} and rodent scrapie infectivity has been demonstrated consistently (Caughey et al., 1997; Lee et al., 2001; Peretz et al., 1997).

The indirect Western blot assay described previously uses the prion-specific monoclonal antibody 3F4 (Kascsak et al., 1987) as the primary antibody, and an alkaline phosphatase-labeled secondary antibody for detection. Although this assay format produces unambiguous results under most circumstances, non-specific signal sometimes interferes with the detection of low concentrations of PrP^{RES}, particularly in samples obtained from immunoglobulin purification processes. This can limit the amount of prion detection, and thus clearance, that can be estimated in these specific processes.

In this paper, the origin of the non-specific signals in the indirect Western blot assay is demonstrated and the development of a one-antibody, or direct, Western blot assay is described. This new assay format uses an alkaline phosphatase-labeled 3F4 Fab fragment for detection, eliminating the need for a secondary antibody. The improvement leads to an approximate 100-fold reduction in the appearance of non-specific signals that interfere with PrP^{RES} detection in immunoglobulin-rich samples, while maintaining the PrP^{RES} detection sensitivity of the assay. An example of the practical application of the direct Western blot assay during the evaluation of the prion clearance capacity of an immunoglobulin purification process is also presented.

2. Materials and methods

2.1. Preparation of 3F4-Fab-alkaline phosphatase conjugate (3F4-Fab-AP)

The anti-PrP monoclonal antibody 3F4 was purified from ascites using a peptide affinity column generated by conjugating a peptide containing the PrP epitope for 3F4, MKHM (Commonwealth Biologicals, Richmond, VA), to a HiTrap™ NHS-activated HP column (Amersham, Piscataway, NJ). The purified antibody was eluted with 100 mM citrate buffer, pH 3.5, and fragmented using immobilized pepsin (Pierce, Rockford, IL) to remove the Fc fragment, generating $F(ab')_2$. Fab fragments were subsequently produced using the reducing agent mercaptoethylamine (MEA; Pierce). Alkaline phosphatase (Biozyme Laboratories, San Diego, CA) was activated using the cross-linker sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC; Pierce). The MEA and sulfo-SMCC were removed from their respective reaction solutions using a D-Salt™ polyacrylamide column (Pierce). Finally, the reduced 3F4-Fab and activated alkaline phosphatase were mixed to produce 3F4-Fab-AP. The resulting conjugate was purified using a HiPrep™ 16/60 Sephacryl S-300 size-exclusion column (Amersham).

2.2. Preparation of hamster scrapie brain homogenate

The preparation of 10% (w/v) hamster scrapie brain homogenate (SBH) (strain 263 K) in Tris-buffered saline was based on a method described previously (Lee et al., 2000). The brain homogenate used in plasma processing clearance studies was clarified by centrifuging 10% SBH for 5 min at $1000 \times g$. The clarified brain homogenate supernatant (cSBH) was transferred to clean vials and stored at $\leq -65\,^{\circ}C$ prior to use. Non-clarified aliquots of 10% SBH were stored in the same manner.

2.3. Sample preparation for Western blot assay format comparisons

Filtrate 2, the product-containing fraction of the IGIV-C process (see Section 2.5) depth filtration step, was obtained from the Bayer production facility. This intermediate has a high immunoglobulin content, and therefore provides an appropriate challenge for comparing the two assay formats. Two 1 mL aliquots of this material were spiked with 1 μL of 10% SBH (non-clarified) and two were left unspiked. One of each of these sample types was treated with 150 μg/mL proteinase K (PK) (Roche, Alameda, CA) for 60 min at 37 °C and the reactions were terminated with 4 mM Pefabloc® SC (Roche), while the other two samples were left untreated. All samples were centrifuged for 60 min at $20{,}000 \times g$ at 4 °C. The supernatants were discarded and the pellets were re-suspended with 100 μL of 2 × SDS sample buffer (Invitrogen, San Diego, CA) and heated 3–5 min at 100 °C. The samples were serially diluted in 0.5 $\log_{10}$ increments in 1 × SDS sample buffer.

2.4. Western blot assays

Two Western blot formats were used in this study: a conventional two-antibody (indirect) assay and a one-antibody (direct) assay using the 3F4-Fab-AP conjugate. The direct Western blot assay procedure is based on the previously described two-antibody system (Lee et al., 2000) with the exception that the two antibodies were replaced by a single conjugate. The different assay formats were performed on the same sample sets for comparison purposes.

The serially diluted samples were electrophoresed on 12% Tris–glycine polyacrylamide gels (Invitrogen) in duplicate and were subsequently electroblotted onto nitrocellulose membranes (Invitrogen). Multimark® multi-colored molecular weight standards (Invitrogen) were also included on each gel. The membranes were blocked for 90–120 min with NAP-Sure Blocker™ (Geno Technology, St. Louis, MO) diluted to 0.5× in Tris-buffered saline containing 0.05%

Tween-20 (TBST; USB, Cleveland, OH). For the indirect Western blot assay, membranes containing each sample type (PK-treated and untreated) were incubated for 60 min at 4 °C in the anti-PrP monoclonal antibody 3F4 (Signet Laboratories, Dedham, MA) diluted 1:5000 in the blocking solution. The membranes were washed with TBST, then incubated overnight (16–20 h) at 4 °C in a goat anti-mouse IgG_{2a}-alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AL) diluted 1:15,000 in the blocking solution. For the direct Western blot assay, membranes with each sample type were incubated overnight in the 3F4-Fab-AP conjugate diluted 1:5000 in the blocking solution.

After the incubation period, all membranes were rinsed five times with TBST and washed 60 min in TBST, followed by an additional 60 min wash in assay buffer (20 mM Tris base, 11.5 mM $MgCl_2 \cdot 6H_20$, pH 10). Excess assay buffer was removed from the membranes by blotting on filter paper and 3 mL of CDP-Star® chemiluminescent substrate containing 150 μL of Nitro-Block-II™ (Applied Biosystems, Foster City, CA) were applied to each membrane for 3–5 min. The excess substrate was removed, the blots were placed in transparent plastic developing folders, and XAR-2 film (Kodak, Rochester, NY) was exposed to the membranes for 60 min, after which the films were developed in a Konica SRX-101 film processor (Konica Medical Corp., Wayne, NJ).

2.5. Generation of immunoglobulin-rich cSBH-spiked process samples from human plasma

Samples containing high concentrations of IgG were generated using the depth filtration step in Bayer's novel process for the production of purified human IgG. The essential steps in the production of the intravenous immunoglobulin product Gamunex®, using caprylate treatment and chromatography (IGIV-C), were previously described (Lebing et al., 2003; Trejo et al., 2003). One of these essential steps, depth filtration, is preceded by the generation of "material in caprylate 2", or MIC-2, by incubating re-suspended Cohn Fraction II + III paste (Cohn et al., 1950, 1946) with caprylate and filtering it through a cloth filter. MIC-2 was obtained from Bayer's Clayton, NC, IGIV-C production facility, and used within 24 h of its generation.

A bench-scale spiking experiment of the IGIV-C depth filtration process was performed to generate samples to test the new direct assay format against the indirect method with samples produced by an actual scale-down. Briefly, the MIC-2 was spiked with cSBH to a final concentration of 0.1% (w/w) and the pH was adjusted to 5.1. Supercel diatomaceous filter aid (World Mineral, Santa Barbara, CA) was added to 2% (w/w). This mixture was pressure-fed directly into the reservoir of a modified CUNO Tri-47 filtration housing and filtered through two Cuno Zeta Plus™ 50CP depth filter pads (CUNO, Meriden, CT) fixed on either side of the reservoir at a pressure not exceeding 20 psi, and rinsed with high purity water. Precipitated material was retained by the filter pads and filter cake, and immunoglobulins were recovered in high yield in the filtrate ("Filtrate 2"), for which the typical IgG content is 4.0–8.2 mg/mL. The experiments were performed in bench-scale volumes (~300 mL). Input, filtrate and resuspended filter cake samples were serially diluted by 0.5 $\log_{10}$ and tested using the indirect and direct Western blot assays.

3. Results

3.1. The origin of non-specific signals in the indirect Western blot assay

When using the indirect Western blot assay (Lee et al., 2001) for prion clearance studies on brain homogenate-spiked plasma fractionation samples, unambiguous results were obtained under most circumstances. However, when immunoglobulin-rich samples were assayed, the detection of low concentrations of specific PrP^{RES} signal (27–30 kDa) would sometimes be obscured by non-specific proteins having an apparent molecular weight of 20–40 kDa (Fig. 1A).

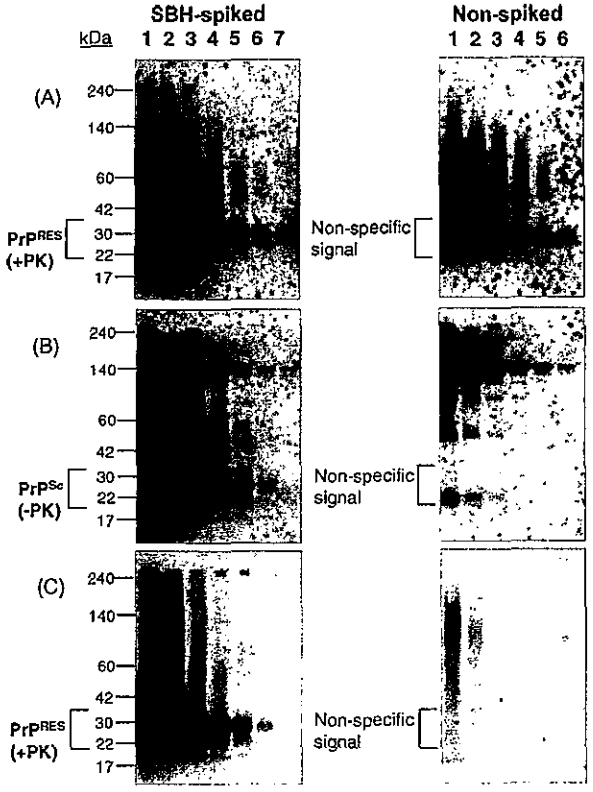

Fig. 1. The origin of non-specific signals and Western blot assay format comparison. Filtrate 2 (human immunoglobulin) samples were either spiked with SBH (final concentration = 0.01%) or left unspiked. The samples were treated with proteinase K (panels A and C: +PK), or left untreated (panel B: −PK). The samples were centrifuged and the pellets were re-suspended with SDS sample buffer. The reconstituted samples were then diluted in 0.5 $\log_{10}$ increments and electrophoresed. After electroblotting the gels onto nitrocellulose, the membranes in panels A and B were processed using the indirect (two-antibody) Western blot assay and the membrane in panel C was processed using the direct (one antibody-conjugate) Western blot assay.

These signals can be reduced by incubating samples with protein G sepharose beads, which removes immunoglobulins (data not shown). Therefore, it is plausible that these signals originate from immunoglobulin fragments generated by PK digestion, a treatment necessary for differentiating between pathogenic PrP^{Sc} and normal PrP^{C}. Indeed, when the PK treatment step was omitted, these signals shifted to ~140 kDa, consistent with the molecular weight of immunoglobulin (Fig. 1B). Furthermore, when PrP^{Sc}-specific primary antibody was omitted from the system, the prion signal from positive samples disappeared, but non-specific signals remained (data not shown). These results indicated that the non-specific signals resulted from interactions between immunoglobulin fragments in the sample and the secondary antibody used in the indirect Western blot assay system.

3.2. Comparison of the direct and indirect Western blot assays

To eliminate the secondary antibody from the assay system, the Fab fragment of the antibody 3F4 was generated and conjugated to alkaline phosphatase to produce a detection probe, 3F4-Fab-AP, that binds directly to the prion protein (Section 2). The 3F4-Fab-AP conjugate was tested in the direct Western blot assay and compared to the indirect Western blot (with labeled secondary antibody) using PK-treated immunoglobulin samples in the presence or absence of a cSBH spike. The use of 3F4-Fab-AP resulted in an approximately 100-fold reduction in non-specific signal for the analyses of immunoglobulin-rich samples (compare the right panels of Fig. 1A and C). The sensitivity of the direct Western was similar to the indirect format (compare the left panels of Fig. 1A and C), with the PrP^{RES} detection endpoints being within 0.5 $\log_{10}$ of one another.

3.3. Application of the direct Western blot assay

To test the direct Western blot assay against the indirect method in a practical setting, both formats were applied to samples from an actual plasma fractionation process, the IGIV-C depth filtration step. The production intermediate sample MIC-2 was spiked with cSBH to a final concentration of 0.1% and processed at bench-scale as described in Section 2. Clarified SBH was used as the spiking material rather than crude brain homogenate to reduce clogging of the filter pads and the resulting pressure spikes. The resulting samples were treated with PK, titrated in 0.5 $\log_{10}$ serial dilutions, and analyzed using the direct and indirect Western blot assay procedures (Fig. 2).

Approximately 4 log PrP^{RES} signal were detected in the input and cake samples assayed by both methods. Non-specific signal through all filtrate dilutions tested (3 log) was observed using the indirect assay, while no signal was seen in the filtrate by the direct method. PrP^{RES} was recovered mainly from the filter cake, and to a much lesser extent, from the filter pads (data not shown), both of which are waste fractions.

4. Discussion

A typical indirect Western blot assay uses a primary antibody to bind a target protein and an enzyme-labeled secondary antibody to bind the primary antibody and form a ternary complex. This complex is capable of generating de-

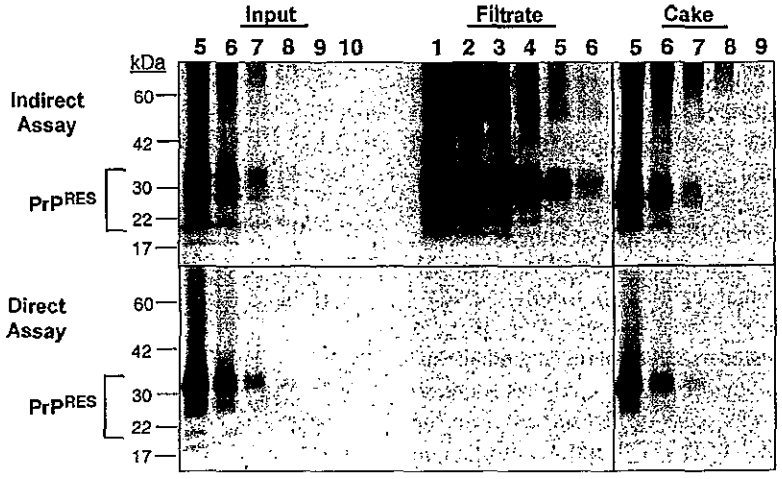

Fig. 2. Comparison of the indirect and direct Western blotting assays testing samples from the IGIV-C depth filtration plasma fractionation step. Samples were generated from the scaled-down model of an immunoglobulin purification process. The starting material MIC2 was spiked with cSBH to a final concentration of 0.1% (Input). The process was performed as described in Section 2. All samples were treated with proteinase K and analyzed using either the indirect or direct Western blot assay. Dilutions loaded are noted under each fraction, and the first four dilutions of the input and cake samples were not loaded, since they were known to be positive for PrP signal.

tectable signal in the presence of a substrate for the enzyme. The secondary antibody is usually produced by immunizing an animal of another species with immunoglobulin preparations derived from the host animal producing the primary antibody. In addition, the host animals for the production of antibodies should be different species than the animal from which the target protein was taken, thus theoretically reducing the potential for cross-reactivity with non-target substances. However, in practice the secondary antibody may still bind some protein components (i.e., immunoglobulins) in the sample containing the target antigen, giving rise to non-specific signal detection. The target protein may sometimes be distinguished from non-specific signals by electrophoretic mobility differences, but this is not always possible, as is illustrated by the case of PrP^{RES} and immunoglobulin fragments after PK treatment of SBH-spiked plasma samples (Fig. 1).

Under most circumstances, the indirect Western blot assay for prion protein detection produces unambiguous results. However, one drawback is its propensity to detect non-PrP signal in samples containing high concentrations of immunoglobulins. In the presence of large amounts of $PrP^{RES/Sc}$ in a given fraction this interfering signal detection is negligible, since it would dilute out before the PrP-specific signal. However, when a brain homogenate-spiked plasma-processing step apparently clears PrP^{Sc} to the limit of detection, it is sometimes difficult to determine whether no detectable PrP^{RES} is present, since the interfering protein bands possess an electrophoretic migration pattern similar to PrP^{RES}. This can result in an underestimation of the clearance capacity for a given process step. Omitting PK treatment reduces the incidence of non-specific bands interfering with accurate PrP analysis, but the question then becomes whether the observed signal is due to PrP^{Sc} (the non-truncated form) or PrP^{C}, since human plasma contains a significant amount of the latter.

The use of the 3F4-Fab-AP conjugate, a "direct" method of performing the Western blot assay, circumvents both problems. It allows PK to be included and significantly reduces the interfering signal, thereby specifically enhancing the determination of the PrP^{RES} content. In addition, use of the conjugate reduces the number of steps and time required to perform the assay, and makes the assay simpler and more economical to use without sacrificing PrP^{RES} detection sensitivity.

The direct Western blot assay was validated in accordance with ICH guidelines and in compliance with GLP regulations on the parameters of repeatability, intermediate precision, sensitivity and limit of detection. The range of detection was 6 $\log_{10}$, using 10% SBH (non-clarified) having an infectivity titer of $\sim 10^9$ IU/mL. The detection endpoint is equivalent to an infectivity titer of about $10^{3.0}$ IU/mL, a value closely approximating that for the indirect Western blot assay (Lee et al., 2000). The specificity was verified by pre-incubating the detection antibody 3F4-Fab-AP with a peptide containing the 3F4 epitope. A 1000-fold suppression in positive signal was observed, indicating that the detection is specific for the prion protein. The repeatability and intermediate precision were assessed by performing the assay with multiple operators on different days. The resulting detection endpoints for PrP^{RES} for these parameters were consistently within 0.5 $\log_{10}$ of one another.

Although a putative case of vCJD transmission by red blood cell transfusion has been reported in the UK (Llewelyn et al., 2004), there has never been a case of classical or variant CJD transmission via plasma products to date. One possible explanation is that the plasma-derived therapeutic proteins are not only devoid of cellular components, which harbor a significant amount of any endogenous infectivity (Brown et al., 1998), but also that the purification processes the plasma undergoes have a high capacity for prion removal (Burnouf and Radosevich, 2003; Cai et al., 2002; Foster et al., 2000; Gregori et al., 2004; Lee et al., 2001; Stenland et al., 2002; Tateishi et al., 2001; Trejo et al., 2003; Van Holten and Autenrieth, 2003). The evaluation of this capacity is typically conducted by performing bench-scale purification processes using PrP^{Sc}-spiked starting material and monitoring prion partitioning using an animal bioassay or Western blot assay. The PrP^{RES} content of various cSBH-spiked and processed plasma fractions, measured using the indirect Western blot assay, has been shown to correlate closely with the infectivity titer measured by animal bioassay (Lee et al., 2001). The bioassay has the advantage of directly measuring infectivity, while the Western blot assay has the advantages of being convenient to perform and producing much faster results (2 days, compared to 8–10 months for the bioassay).

After initial studies indicating that the direct Western blot assay reduced the incidence and intensity of non-specific protein signal, and validation of the assay, the direct Western blot assay was used to test SBH-spiked samples in the IGIV-C depth filtration fractionation step. Determination of PrP^{Sc} clearance by this step has been problematic in the past due to a combination of the high immunoglobulin concentration in the product-containing filtrate and the very low content of PrP^{Sc} remaining in this fraction after processing the spiked starting material. Similar observations have been reported in other studies where immunoglobulins were present in the samples assayed (Field, 2004; Serban et al., 2004). However, use of the direct Western blot assay method circumvented this problem entirely in the case of the IGIV-C depth filtration clearance study presented here (Fig. 2).

The elimination of the secondary antibody from the Western blot assay is invaluable for evaluating the prion clearance capacity of processes used to purify various plasma-derived therapeutic proteins, especially IgG-rich fractions. The prion-specific conjugate will also be useful in other circumstances, such as for the evaluation of prion clearance of recombinant therapeutic monoclonal antibodies, or the detection of prions in other samples where non-specific signal is observed (Furukawa et al., 2004).

Acknowledgements

The authors wish to acknowledge Dr. Linda Anderson of Bayer Diagnostics (Elkhart, IN) for her invaluable advice concerning the preparation, purification, and use of conjugated antibodies, and Eileen Hawkins, Michael Paisley, and Rosmy Valoyes for their help in obtaining reagents. We thank Dr. Richard Rubenstein for providing antibody 3F4.

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