Short Communication

Improved conformation-dependent immunoassay: suitability for human prion detection with enhanced sensitivity

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The presence of pathogenic prion protein (PrP^{Sc}) in lymphoid tissues of variant Creutzfeldt–Jakob disease (vCJD) patients raises questions as to whether prions may be present in bodily fluids as well. Currently, transgenic mice are highly sensitive *in vivo* tools for the study of prions in tissues or fluids containing high levels of normal prion protein (PrP^{C}). We report here an *in vitro* assay with virtually equivalent sensitivity incorporating a capture antibody into a sandwich conformation-dependent immunoassay (CDI), resulting in 30- to 100-fold increased sensitivity compared with the original, direct CDI. Furthermore, spiking plasma with vCJD prions in different preparations demonstrated that sandwich CDI detects prions with different biophysical properties at high sensitivity, even without proteinase K pretreatment of samples. Thus, sandwich CDI represents a powerful tool to study prions in bodily fluids of CJD/vCJD patients, with a turnaround time of less than 24 h.

Prions, the causative agents of Creutzfeldt–Jakob Disease (CJD) and related neurodegenerative disorders in animals and humans including variant CJD (vCJD), have never been detected in human blood and have never been transmitted by blood or plasma products. Nevertheless, the presence of prions in lymphoid tissues of patients afflicted with vCJD (Hill *et al.*, 1997) and detection of a protease-sensitive, pathogenic prion protein (PrP^{Sc})-like form of prion protein (uPrP) in urine of prion-infected hamsters (Shaked *et al.*, 2001) have intensified the search for assays suitable for studying body fluids. Since infectivity bioassays using primates or normal or transgenic mice take several years, more rapid *in vitro* assays with similar sensitivity are needed. In addition, knowledge of the biophysical properties of the theoretical prion contaminant in blood or plasma, e.g. degree of protease-resistance, is completely lacking. Prion testing is further complicated because there are no diagnostic reagents that discriminate between PrP^{Sc} and its normal isoform, PrP^{C}, which is often present in excess and cross-reacts with all antibodies used for prion screening. We have evaluated the suitability of a conformation-dependent immunoassay (CDI) for *in vitro* testing of bodily fluids for human prions utilizing a capture antibody; this sandwich CDI was based on the previously reported ELISA-formatted, time-resolved fluorescence (TRF) CDI (Safar *et al.*, 1998). The CDI included a prion concentration step and corrected for background signals caused by PrP^{C} by measuring binding of an antibody to PrP whose epitope is accessible in PrP^{C} and hidden in non-denatured PrP^{Sc} but accessible after denaturation (Safar *et al.*, 1998).

Monoclonal antibody (mAb) 1120-64-9 and mAbs 1120-63-33 and 1120-2214 were produced as described (Peters *et al.*, 1985): one 6-week-old BALB/c mouse was immunized with minute amounts of human PrP^{C} immunopurified from brains of transgenic (tg) mice expressing human PrP by intraperitoneal and, before recovery of the spleen, by intravenous injections with a total of five boost injections several weeks apart. The resulting hybridoma cells were deposited at DSMZ (Germany) as ASM DCC 2523 (and ASM DCC 2522 and 2524, respectively).

A 10 % (w/v) vCJD brain homogenate, prepared in PBS, was diluted in human plasma in 0·5 $\log_{10}$ steps and Sarcosyl (20 %, w/v, stock solution in PBS) was added to a final concentration of 2 % and processed according to Safar *et al.* (1998). After incubation of the diluted samples at 37 °C for 15 min, phosphotungstic acid (PTA) was added to a final concentration of 0·32 % (w/v). Samples were then incubated at 37 °C and precipitates collected by centrifugation at 14 000 ***g*** for 30 min at room temperature. Pellets were further resuspended in H_2O/0·2 % Sarcosyl and split into two aliquots: one aliquot was denatured by adding guanidinium hydrochloride (4 M final concentration) and heating at 83 °C for 6 min, while the other aliquot was left untreated. Both aliquots were diluted and adjusted to a final guanidinium concentration of 0·205 M. For the

originally reported direct CDI, microtitre plates were pre-activated with glutaraldehyde. For the modified sandwich CDI, microtitre plates were coated with 10 µg mAb 1120-64-9 ml^{-1}, which binds specifically to human PrP. Denatured and native aliquots were split and loaded in triplicate on to the plates. PrP bound either by glutaraldehyde or by mAb was detected using Europium-conjugated mAb 3F4 as described (Safar *et al.*, 1998) and TRF counts of the denatured aliquots were divided by the counts of corresponding native aliquots to give the d/n ratio. The cut-off value was calculated from the mean d/n ratio of at least 12 non-spiked plasma samples plus three times the standard deviation.

In the direct CDI procedure, binding of PrP^{Sc} to the plate was not very efficient and, as a result, higher than cut-off d/n ratios were recorded up to 10^{-2} dilutions. In the sandwich CDI, positive d/n ratios were recorded at dilutions higher than 10^{-4} (Fig. 1a) because the first antibody specifically enriched prion proteins on the plate. Pretreating the vCJD-spiked plasma samples with 250 µg proteinase K (PK) ml^{-1} for 60 min, followed by addition of 1 mM PMSF to stop digestion before addition of PTA, further increased the sensitivity of the sandwich CDI by greater than threefold (0·5 $\log_{10}$), because background signals resulting from co-precipitated PrP^{C} were further eliminated by enzymatic digestion (Fig. 1a). Thus, the sandwich CDI detected vCJD with 100-fold higher sensitivity in plasma without protease treatment than the direct CDI.

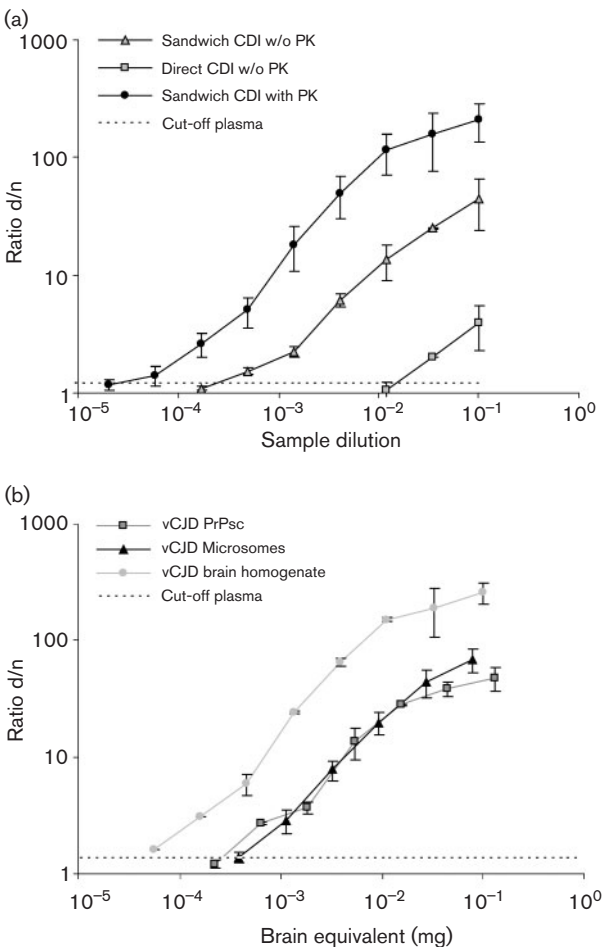

Fig. 1. (a) Detection of variant CJD brain homogenate spiked in plasma using different CDI formats. (b) Detectability of different variant CJD preparations in plasma using sandwich CDI.

The biophysical nature and the degree of membrane association of the theoretical prion contaminants in plasma are not known due to the lack of prion detection in blood or plasma of CJD/vCJD patients. In order to test whether the sandwich CDI could still detect PrP^{Sc} when it was present in different membrane microenvironments, we spiked plasma with PrP^{Sc} in the form of crude brain homogenates, microsomal membranes and purified, non-membrane-bound PrP^{Sc} isolated from vCJD brain. The three spike preparations were derived from equivalent amounts of brain, but preparation of microsomes and purified PrP^{Sc} resulted in ~90 % loss of PrP^{Sc} present in the starting material (Bolton *et al.*, 1987), as reflected in the shift of the respective curves by about 1 $\log_{10}$. All three spike forms were detected in plasma with high efficiency to dilutions around 10^{-4} (Fig. 1b).

In order to allow comparison of the sandwich CDI sensitivity with other established immunoassays, WHO CJD/vCJD reference samples were sequentially diluted in human plasma, treated with PK and analysed using the sandwich CDI. The WHO reference samples included one sporadic or sCJD type 1, one sCJD type 2 and one vCJD brain homogenate (Minor *et al.*, 2000). The experiments were performed by three independent operators on two different days. Immunoreactivity endpoints were used to calculate the relative CDI titres ml^{-1} (CDI-$Unit_{50}$ ml^{-1}) of brain homogenate (based on an input of 167 µl brain homogenate per test), resulting in similar PK-resistant PrP^{Sc} concentrations for sCJD type I and the vCJD brains of $10^{6·0}$ CDI-$Unit_{50}$ ml^{-1} and $10^{6·2}$ CDI-$Unit_{50}$ ml^{-1}, respectively (Table 1). The sCJD type 2 sample showed 10-fold lower PrP^{Sc} concentrations (CDI titre $10^{5·0}$ CDI-$Unit_{50}$ ml^{-1}). These results demonstrated that sandwich CDI was able to detect sCJD as well as vCJD. This study further showed that 1 nl of the 10 % vCJD and sCJD type 1 brain homogenates, respectively, was the minimum volume detectable in a single microtitre well by sandwich CDI.

Endpoint titration studies of sCJD brain homogenates in highly susceptible tg mice expressing human PrP^{C} indicate that CJD brain homogenates contain up to 10^{6} ID_{50} ml^{-1} (J. Safar, personal communication). Thus, the existing data suggest that sandwich CDI has a similar sensitivity for PrP^{Sc} detection as infectivity bioassays in highly susceptible tg mice using the most effective route of intracerebral inoculation of prions: 1 nl of such a brain homogenate contains 1 ID_{50} as well as 1 CDI-$Unit_{50}$. Preliminary data from an ongoing study directly comparing sandwich CDI with endpoint titration of sCJD prions in tg mice has

Table 1. Dilution endpoint titres in WHO CJD reference samples using the sandwich CDI

The relative CDI titre (CDI-Unit$_{50}$ ml^{-1}) was calculated using Spearman–Kärber statistical analysis.

	No. of positives/total			
Dilution	Control (RU 97/03)	Sporadic CJD MM Type 1 (RU 99/009)	Sporadic CJD MM Type 2 (RU 97/008)	New variant CJD MM (RU 98/148)
$10^{-1\cdot0}$	0/4	6/6	6/6	6/6
$10^{-1\cdot5}$	0/4	6/6	6/6	6/6
$10^{-2\cdot0}$	0/4	6/6	6/6	6/6
$10^{-2\cdot5}$	0/4	6/6	6/6	6/6
$10^{-3\cdot0}$	0/4	6/6	6/6	6/6
$10^{-3\cdot5}$	0/4	6/6	6/6	6/6
$10^{-4\cdot0}$	0/4	6/6	5/6	5/6
$10^{-4\cdot5}$	0/4	6/6	1/6	6/6
$10^{-5\cdot0}$	0/4	3/4	0/4	4/4
$10^{-5\cdot5}$	0/4	1/4	0/4	2/4
CDI-Unit$_{50}$ ml^{-1}*	NA	6·0 $\log_{10}$	5·0 $\log_{10}$	6·2 $\log_{10}$

*0·167 ml brain homogenate used.
NA, Not applicable.

confirmed that sandwich CDI is at least as sensitive as this tg mouse bioassay system (Safar, 2003). Even without PK treatment of samples, the sandwich CDI would have a sensitivity near to the endpoint titration bioassay in transgenic mice, making it possible to detect minute amounts of protease-sensitive PrP^{Sc} aggregates in biological fluids if they were present at all. We are currently evaluating vCJD samples using the CDI and appropriate tg mice in parallel for direct comparison of their sensitivities. The WHO collaborative study will coordinate evaluation of the CJD/vCJD reference samples described earlier in bioassay systems, including tg mice. These calibration studies will allow us, for the first time, to establish a detection limit for the CDI and other human prion immunoassays in terms of units of infectivity. In a recent study using a sandwich CDI employing recombinant antibody fragments for bovine PrP, the sensitivity was similar to that determined by endpoint titration in tg mice expressing bovine PrP (Safar *et al.*, 2002).

Despite reported evidence for the appearance of very low levels of prion infectivity in blood or plasma during the clinical phase of prion disease in experimental rodent models (Brown *et al.*, 1998, 2001; Cervenakova *et al.*, 2001) and preliminary data on transmission of prion disease by transfusion in sheep (Houston *et al.*, 2000; Hunter *et al.*, 2002), transmission of CJD/vCJD by transfusion has never been documented in humans. Epidemiological studies (Baron & Prusiner, 2000) as well as failed attempts to transmit sporadic CJD prion disease to animals by blood transfusion (Brown *et al.*, 1994) suggest that prions either are not present in blood of sCJD patients or are there at levels that are insufficient to elicit prion infection following transfusion. However, with the occurrence of prions in lymphoid tissues of vCJD patients and the limited information on epidemiology of vCJD, the theoretical risk of vCJD transmission by blood or blood products, though never substantiated, is more difficult to rule out at this time. Although prions could not be detected in buffy coats or plasma from vCJD patients using PTA precipitation and immunoblotting (Wadsworth *et al.*, 2001) or infectivity bioassays (Bruce *et al.*, 2001), questions as to the sensitivity of the methods and the biophysical properties remain. The degree of PK resistance of the theoretical prion contaminant in blood or plasma could not be assessed when procedures such as PK pretreatment of samples were used in prion detection assays (Wadsworth *et al.*, 2001). The potential presence of a PK-sensitive, PrP-like protein in the urine of prion-infected animals, which becomes protease-resistant after dialysis, has been reported (Shaked *et al.*, 2001). This uPrP could be blood-derived and might only be identified using procedures without prior proteolysis of samples. Due to the sandwich CDI's ability to detect low levels of PrP^{Sc} in the presence of 1000 times higher concentrations of PrP^{C}, even without the use of PK, such questions can now be addressed by evaluating tissues and body fluids of clinically affected vCJD patients for PrP^{Sc} distribution. Only if PrP^{Sc} can be reproducibly detected in blood, buffy coat or plasma by the sandwich CDI in persons who later develop (v)CJD could plasma screening be considered. Implementation of a plasma screening system requires a true positive control for validation of the assay. The closest substitute for a patient's blood might be endogenous infectivity in CJD-inoculated transgenic mice as described for human prions adapted to wild-type mice (Brown *et al.*, 1998). The CDI is currently being used to evaluate the prion clearance capacity of purification and concentration steps used in the manufacturing processes of plasma-derived products (Vey *et al.*, 2002).

In order to determine the human PrP epitope to which mAb 1120-64-9 binds, overlapping 15-mer peptides covering the

PrP 90–231 sequence were tested using this mAb in an ELISA-formatted assay with negative results (data not shown), indicating that the epitope is not a linear peptide sequence, although mAb 1120-64-9 can detect human prions in Western blots (data not shown). Furthermore, other covalent modifications present on native, mammalian-derived PrP, such as *N*-linked glycosylation or C-terminal GPI-anchor linkage, do not seem to be part of the epitope because human recombinant PrP 90–231 was detected by mAb 1120-64-9 (data not shown). The only covalent modification that could be involved in antibody binding was the disulphide bridge connecting cysteine-179 with cysteine-214. Antibody binding to human PrP was tested in the absence or presence of the disulphide bridge by treatment of purified human PrP^{Sc} with 4 M guanidinium hydrochloride in the absence or presence of 3·3 mM DTT for 6 min, prior to binding it to a microtitre plate and detection with 1120-64-9 and a europium-labelled rabbit anti-mouse IgG antiserum. After reduction, parallel samples were treated with iodacetamide (10 mM final concentration) in order to avoid reoxidation of the disulphide bridge. DTT-treated PrP resulted in a low 1120-64-9 binding signal, in contrast to untreated PrP (Fig. 2). Furthermore, efficient binding of 1120-64-9 to PrP could be restored after reoxidation of reduced samples (not treated with iodacetamide) using oxidized glutathione or incubation at room temperature. From these experiments, we concluded that the epitope to which mAb 1120-64-9 binds depends on a correctly formed disulphide bridge. Previous studies have characterized linear and conformational epitopes on prion protein (Li *et al.*, 2000) but this is the first time that an epitope has been linked to the presence of the disulphide bridge, suggesting that the disulphide group itself could be an integral part of the peptide sequence or could form a conformational epitope by holding together otherwise distantly located amino acid groups.

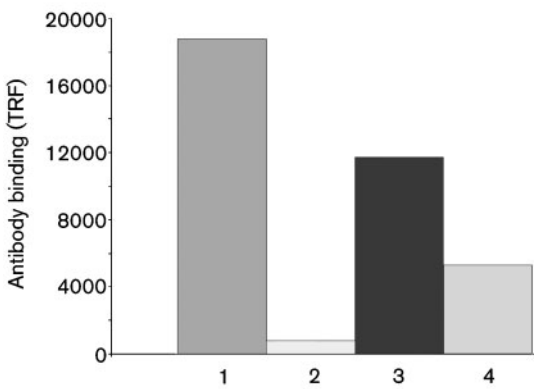

Fig. 2. Reduction of the disulphide bridge in PrP protein diminishes the binding of mAb 1120-64-9. Column (1), PrP^{Sc} not treated with DTT (control); (2), PrP^{Sc} treated with DTT and iodacetamide; (3), PrP^{Sc} treated with DTT and oxidized glutathione; (4) PrP^{Sc} treated with DTT and incubated at room temperature for 3 h in solution.

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