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PROPOSAL TO ESTABLISH WHO REFERENCE REAGENTS FOR *IN VITRO* ASSAYS OF CJD SPECIMENS

WHO Working Group on International Reference Materials for Diagnosis and Study of TSEs

SUMMARY

Under the direction of the WHO Working Group on International Reference Materials for Diagnosis and Study of TSEs, the WHO has sponsored the preparation of candidate reference materials consisting of 10% w/v homogenates of brain from 1 normal human, 2 sporadic cases of CJD, and 1 case of vCJD. All cases were homozygous for methionine at codon 129 of the PrP gene. Six laboratories assayed the homogenates for relative concentration of PrP amyloid by Western blot. The ranking of the concentration was similar for five of the participants with concentrations 1 to 3 times greater than for the vCJD sample for one of the

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sCJD homogenates and 3 to 10 fold less than vCJD for the other sCJD sample. Despite differences in technical detail the minimum detectable volume of homogenate from the vCJD infected brain was very similar for all laboratories at 50 to 200 nl, irrespective of the extent to which it was diluted. Differences in the detectable end point dilution, depended only on the proportion of the original sample actually processed and loaded for assay. The materials are under evaluation by other *in vitro* assays and infectivity assays *in vivo*. The sporadic cases were selected, on the basis preliminary local sampling of the brains, to represent two different common types based on the glycoform patterns of the PrP amyloid on Western blots. In both cases when larger, 100g, samples of brain were homogenized, both glycoform types were found to be represented. The glycoform pattern for vCJD was unique to vCJD and remained unchanged in the 100g sample.

It is proposed that the preparations be given the interim status of WHO Reference Reagents. The materials are stored at -86^{o} in aliquots of 0.5ml.

INTRODUCTION

Creutzfeldt-Jakob disease is a transmissible spongiform encephalopathy (TSE [1]), a class of disease characterised by slow progressive non-inflammatory neurodegeneration with a long clinically silent period before symptoms develop. Currently, infectivity can only be demonstrated by infection of susceptible animals. However, in the course of the disease a normal host protein (PrP) is deposited in an abnormal amyloidotic form that is resistant to proteolysis, (PrP^{res}), and which is believed by many to be the infectious agent itself (2). Whether or not PrP^{res} is the agent, detection of PrP^{res} is pathognomonic for the presence of infectivity and PrP^{res} concentration has generally correlated well with infectivity levels. Thus, there are numerous efforts to establish reliable assays for the presence and concentration of PrP^{res} as an indication of infection and infectivity concentration. These efforts would be greatly facilitated by the existence of international reference materials to enable reliable comparisons between laboratories of quantifiable parameters of assay performance.

The PrP molecule contains two glycosylation sites and the distribution of the PrP^{res} molecule between the un-, mono-, and diglycosylated forms has been used to distinguish subtypes of TSE disease. However, at least two different classification systems are in use and the pattern differences on which they are based, as distinguished by Western blot, can be quite subtle. Thus, there is also great interest in common reference materials with which to calibrate typing efforts.

As a first step in establishing a comprehensive collection of reference materials in support of TSE research and assay development, the WHO Working Group on International Reference Materials for the Diagnosis and Study of Transmissible Spongiform Encephalopathies commissioned the preparation of four reference materials prepared from human brain. Homogenates were prepared from 1 normal human, 2 sporadic cases of CJD, and 1 case of vCJD and distributed in 0.5ml aliquots in 2000 ampoules each. In studies organized by the Working Group, the preparations were characterised with respect to PrP^{res} content by several variations of the Western blot assay. The Western blot study also afforded an opportunity to compare the PrP^{res} glycoform type of the preparations.

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MATERIALS AND METHODS

Preparation of brain samples

Samples of grey-matter-enriched frontal cortex from four human brains were provided by the CJD Surveillance Unit in Edinburgh. All were from patients homozygous for methionine at codon 129 of the prion-protein-encoding (PRNP) gene. This is the most prevalent PrP allele in those populations in which it has been investigated and it also shows an even higher representation in sCJD cases compared to the general population (3). All cases of vCJD to date have been in persons of this genotype. The disease had been confirmed histologically in all cases. Samples of the infected brains had previously been shown to contain PrP^{res} and the two sCJD cases were selected on the basis of their PrP^{res} glycoform patterns as representing the two most common glycoform subtypes. The variant CJD specimen, had transmitted disease on inoculation of animals (J Ironside, unpublished). Samples of approximately 100g were provided from one normal non-CJD brain, one sporadic CJD brain, nominally of the first common glycotype (sp1CJD) one sporadic CJD brain nominally of the second common glycotype (sp2CJD) and one variant CJD brain (vCJD). The specimens were homogenised in 0.25M unbuffered sucrose using a programmable Camlab Omni-Mixer set at 4000 rpm for six bursts of 30 seconds to give a final volume of 1 litre of 10% homogenate. A new grinder was used for each brain to avoid the possibility of cross contamination. Analysis of an uninfected bovine brain homogenised by this method showed a very uniform distribution of particle size (data not shown). The suspension was kept on ice on a magnetic stirrer; and dispensed in 0.5 ml aliquots into 2000 1.2 ml cryovials (Nalgene); flash frozen in liquid nitrogen; and stored at -86°C for subsequent use. As the preparation is a liquid fill, no weights and range were determined. The bioburden was 200 to 600 cfu per ml.

Design of study to determine relative concentration of PrP^{res} in the candidate reference materials

Participants were requested to use routine in-house immunoblot methods to titrate the preparations four times on separate vials, using ten-fold dilutions for the first run to establish approximate end points and three-fold serial dilutions for the remainder. The final dilution containing detectable material was reported and raw data returned with technical details of the method employed.

RESULTS

Six laboratories participated in the collaborative study. Two others stopped after preliminary characterisation showed that the sporadic CJD samples were mixtures of type 1and type 2 glycotypes. The procedures followed by those laboratories returning data are summarised in Table 1. They involved treatment of the samples with proteinase K at either 50 or 100 micrograms per ml for 30 to 60 minutes and detection of undigested PrP, identifed as PrP^{res}, after electrophoresis and immunoblot using either the PrP specific 3F4 (4) or 6H4 (5) monoclonal antibodies. The sample volume loaded ranged from 6 to 40 microlitres but, because of differences in pre-treatment and dilution or concentration, the equivalent volume of brain homogenate which would have been loaded from a hypothetical undiluted preparation varied from 3 to 100 microlitres as shown in the table. The other major difference in reported techniques was in the dilution of the samples. One laboratory diluted the sample in buffer before digestion, two diluted the sample in normal brain suspension before digestion, and three diluted the sample in buffer after digestion.

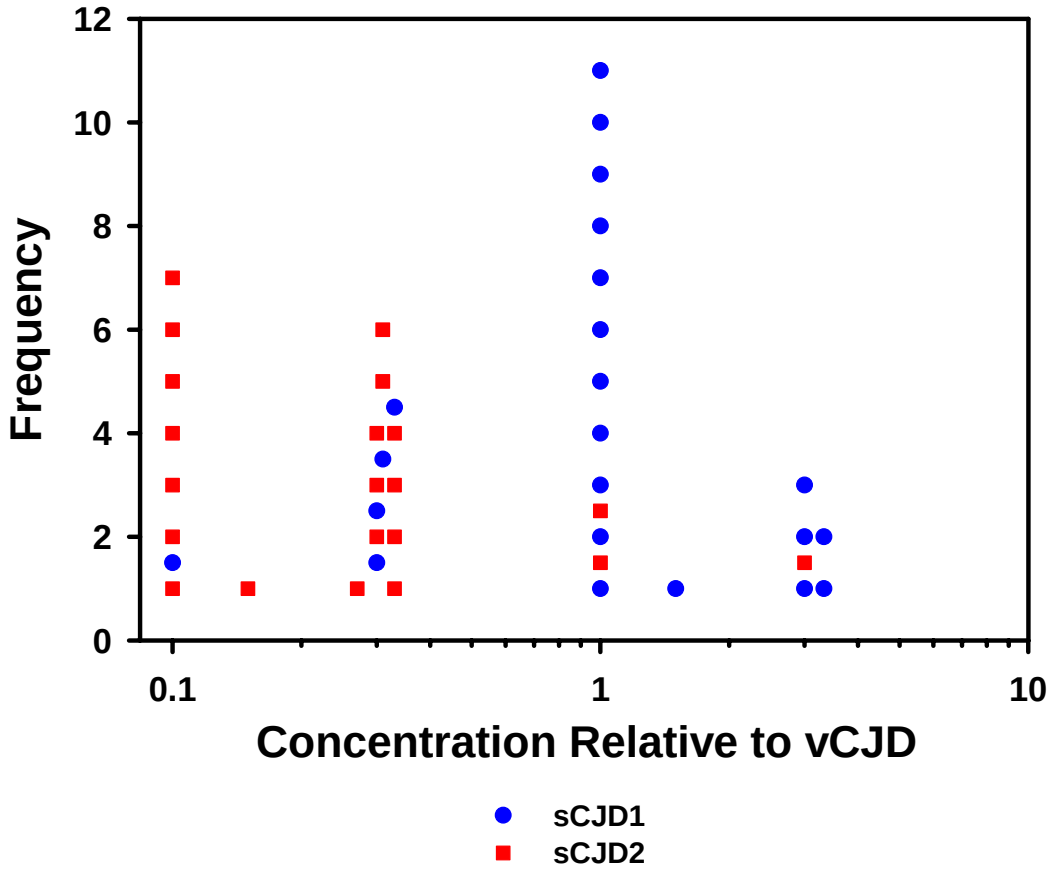
The end point dilutions, defined as the last dilutions giving a positive signal, are summarised in Table 2A, with the geometric mean titres of each group of four determinations. The variation in the titre of a given preparation within a laboratory was of the order of three-fold, which was the dilution interval, with occasional outliers. The range of end point titres between laboratories was much higher, reflecting differences in pre-concentration and loading (see below).

In Table 3 the endpoints are expressed in terms of the minimum volume of original brain homogenate detectable, by dividing the volume loaded from an undiluted preparation by the geometric mean of the end point dilutions. The variation in the detection limit between laboratories for the different samples was 4.9 fold (sp1CJD), 4.4 fold (spCJD2) and 9.0 fold (vCJD) where the dilution interval was 3 fold. Variations in the details of the methods, in particular dilution of the sample into normal brain before digestion, appeared to have no effect on the assay sensitivity.

The relative concentrations of PrP^{res} among the three CJD preparations was calculated separately for each determination from each laboratory by dividing each of the sCJD endpoints by the vCJD endpoint (see Table 2B attached-Excel spreadsheet for the calculations). Each determination was analysed individually as there can be large differences in the absolute values of the endpoints between determinations related only to the exposure conditions which will have no effect on the relative end point dilutions within the determination. The results when plotted as a frequency distribution of the values obtained, Fig. 1, showed the first sporadic sample, sCJD-1, to have a PrP^{res} concentration roughly equal to that of vCJD and the second sporadic sample, sCJD-2, to have one third to one tenth the concentration of the other two when assayed by either 3F4 or 6H4 antibodies.

The geometric mean of the potencies of the preparations determined in each laboratory relative to the vCJD sample are shown in Table 4.

Fig. 1 Distribution of Relative Concentration Determinatior



Characterisation of materials

Creutzfeldt-Jakob Disease is sub-classified based on host PrP genotype clinical presentation and at least two different classifications of the patterns of glycosylation of PrP^{res} at its two glycosylation sites (6,7). Except in rare cases, a brain from a case of sporadic CJD will typically have only one glycoform type present, although there have also been reports of multiple types in a single brain (8, 9). The sporadic cases selected for these reference materials were chosen on the basis of preliminary analysis of small samples showing that one contained the most common glycoform type and the other the second most common glycoform type. However results returned from laboratories 1 to 6 as well as information from the two additional laboratories who carried out preliminary studies showed that both preparations contained a mixture of the two most common glycoform types.

DISCUSSION

International reference materials with which to calibrate laboratory working stocks of TSE infected materials will greatly facilitate the development of diagnostic procedures for TSEs and other TSE research by providing the basis for direct comparison of results between laboratories. The various assays currently under development to detect PrP^{res} by immunological or other methods could be directly compared by reference to a common standard. The relative susceptibilities of the numerous strains of transgenic mice that have been developed to assay infectivity directly from human tissues could be directly compared. To date, cross comparisons of results between laboratories using the same specimens has been rare and idiosyncratic. The preparations described here have been produced in sufficient quantity to serve as calibrants for the in house stocks from a large number of laboratories and will be available to any laboratory qualified to work on TSEs that requests them.

The initial characterisation of the candidate reference materials reported here was based on immunoblots. Differences in sample preparation and the volumes loaded onto the gels resulted in a one-hundred-fold range of detectable endpoint dilutions between laboratories assaying the same material. . Within laboratories reproducibility was within one dilution interval, or on the order of three-fold. . When the results were expressed in terms of minimum detectable volume of homogenate, the results between laboratories were surprisingly uniform with a range of 4 to 9 fold. This is of the same magnitude as the dilution steps employed and is similar to the within laboratory variation. It was striking that the details of the assays, specifically dilution before or after digestion in brain or buffer, had no detectable influence on the results obtained. This supports the general robustness of the Western blot methodology and points to the antibody/antigen interaction as an irreducible limitation on the sensitivity of the assay. All participants utilize d either the 3F4 or 6H4 antibodies with very strong and similar avidities.

The observation that both sporadic CJD brain preparations contained a mixture of the most common PrP^{res} glycosylation patterns could not be explained in terms of either mixing of samples from more than one individual or gel artefacts, and is interpreted to mean that the patients' brains contained both types. This may be either because both brains were atypical or because brains of patients with sporadic CJD contain both types of PrP^{res} more commonly than had been thought. Even when small tissue samples (<100mg) are assayed two labs have independently reported occasionally finding both glycoform types in individual sCJD brains (10,11). The specimens used to prepare the reference materials were 100g each, a brain mass 1000 times larger than is usually examined. In contrast, the vCJD brain preparation contained only the distinctive glycoform pattern that is characteristic of the bovine derived disease. While the reproducible behaviour of the two sporadic brain preparations in the Western blot study suggests that the mixture of types does not present a problem for those assays, the suitability of the materials from the sporadic CJD cases as reference preparations for other purposes can be questioned, and was the reason why two laboratories stopped work after preliminary characterisation. The prevalence of PrP^{res} glycoform mixtures will only be settled by examination of larger numbers of specimens in the 100g range or by more extensive sampling from such specimens. Further studies using other methods including infectivity assays, are currently underway or planned. The materials described here and others in development are being further examined by a range of procedures, enabling a correlation between *in vitro* and *in vivo* concentrations and providing well characterised materials by

which new procedures and reagents, including new lines of transgenic animals, can be compared.

These materials are intended for use in small quantities as calibrants for laboratory working stocks, not as seed materials or for use in spiking studies or as routine control materials, and are now available for this purpose. It is intended to expand the available materials to include brain preparations from sCJD cases showing only one glycotype pattern and cases representing other PrP genotypes. Lymphoid tissues from CJD and vCJD are also being developed as reference materials. The committee is also exploring various options for supplying reference materials to facilitate the development and evaluation of blood based TSE assays. Finally, reference materials utilizing rodent brain, blood and lymphoid tissues are being developed to support the most commonly used animal models for TSE research.

TABLE 1

Summary of methods used in immunoblot assays

Lab	Pre-treatment	Proteinase K Treatment	Post-treatment	Volume loaded	Equivalent volume loaded	Gel Concentration (1)	Detector antibody
1	Thaw, vortex	50µg/ml, 37°, 1h	Dilute in 2% SDS, boil 10 min	5µl	10µl	16%	3F4
2	Dilution in lysis buffer	50µg/ml, 37°, 1h	Boil in 2% SDS, 10 min	40µl	20µl	12%	3F4
3	Dilution in 10% brain	50°, 30 min (2)	---	6µl	3µl	12%	6H4
4	Thaw, homogenise	100µg/ml, 37°, 1h	Dilute in 2% SDS Boil in 2% SDS	15µl	8µl	14%	3F4
5	Thaw, homogenise	50µg/ml, 37°, 1h	Boil in 2% SDS, 10 min, Dilute in 2% SDS	10µl	5µl	12%	3F4
6	Dilution in normal brain. Pellet 500µl sample to digest	100µg/ml, 37°, 30 min	Boil in 2% SDS, 10 min	34µl	100µl	12%	6H4

(1) Equivalent volume of brain homogenate which would be considered undiluted. All laboratories used enhance chemiluminescence detection (ECL)
(2)Concentration of proteinase K in a proprietary commercial digestion mixture was not specified. A proprietary loading buffer was also used

TABLE 2A

End point titres detectable by immunoblot

Lab	sp1CJD	GMT	Sp2CJD end-points	GMT	vCJD endpoints	GMT
1	10, 30, 10, 100	23	10, 10, 10, 10	10	100, 100, 30, 30	55
2	100, 100, 100, 100	100	10, 10, 10, 10	10	100, 100, 100, 100	100
3	9, 3, 9, 81	12	9, 0, 1, 9	3	3, 3, 3, 27	5
4	100, 100, 100, 300	120	10, 30, 30, 30,	23	100, 100, 100, 200	120
5	10, 11, 36, 11	15	1, 3, 11, 11	4	10, 11, 36, 36	20
6	1000, 300, 1000, 300	550	300, 300, 100, 300	225	300, 1000, 1000, 300	550

WORLD HEALTH ORGANIZATION
ORGANISATION MONDIALE DE LA SANTE

WHO/BS/03.1965
Rev. 1

Table 2B

Relative concentration of PrP amyloid in the WHO candidate TSE reference materials

Concentration of PrP^{res} in sCJD-1 and sCJD-2 relative to vCJD

Calculated for each separate determination

Determination	End Point Dilutions				Concentration Relative to vCJD		
Laboratory 1.	sCJD-1	sCJD-2	vCJD		sCJD-1	sCJD-2	vCJD
1	10	10	100		0.10	0.10	1.00
2	30	10	100		0.30	0.10	1.00
3	10	10	30		0.33	0.33	1.00
4	100	10	30		3.33	0.33	1.00
GMT	23	10	55	from GMT	0.43	0.18	1.00
				Ave.	0.24	0.22	1.00
Laboratory 2.							
1	100	10	100		1.00	0.10	1.00
2	100	10	100		1.00	0.10	1.00
3	100	10	100		1.00	0.10	1.00
4	100	10	100		1.00	0.10	1.00
GMT	100	10	100	from GMT	1.00	0.10	1.00
				Ave.	1.00	0.10	1.00
Laboratory 3.							
1	9	9	3		3.00	3.00	1.00
2	3	0	3		1.00	0.00	1.00
3	9	1	3		3.00	0.33	1.00
4	81	9	27		3.00	0.33	1.00
GMT	12	3	5	from GMT	2.28	0.58	1.00
				Ave.	2.50	0.33	1.00
Laboratory 4.							
1	100	10	100		1.00	0.10	1.00
2	100	30	100		1.00	0.30	1.00
3	100	30	100		1.00	0.30	1.00
4	300	30	200		1.50	0.15	1.00
GMT	132	23	119	from GMT	1.11	0.19	1.00
				Ave.	1.13	0.21	1.00
Laboratory 5.							
1	10	1	10		1.00	0.10	1.00
2	11	3	11		1.00	0.27	1.00
3	36	11	36		1.00	0.31	1.00
4	11	11	36		0.31	0.31	1.00
GMT	14	4	19	from GMT	0.74	0.22	1.00
				Ave.	0.83	0.25	1.00
Laboratory 6.							
1	1000	300	300		3.33	1.00	1.00
2	300	300	1000		0.30	0.30	1.00
3	1000	100	1000		1.00	0.10	1.00
4	300	300	300		1.00	1.00	1.00
GMT	548	228	548	from GMT	1.00	0.42	1.00
				Ave.	1.41	0.60	1.00

TABLE 3

Sensitivity of immunoblot assays

Laboratory	Equivalent Volume loaded[(1)]	sp1 CJD[(2)]	sp2 CJD	VCJD
1	5	0.21	0.5	0.09
2	20	0.2	2	0.2
3	3	0.25	1.0	0.60
4	8	0.067	0.47	0.067
5	5	0.33	1.2	0.25
6	100	0.18	0.44	0.18

(1) Equivalent volume (µl) of 10% brain homogenate in undiluted sample
(2) Equivalent volume (µl) of 10% brain homogenate in loading last detectable track

TABLE 4

Relative concentration of PrP^{res} in the sporadic CJD Specimens compared to the vCJD Specimen

Laboratory	sCJD1	sCJD2
1	0.42	0.18
2	1.0	0.1
3	2.4	0.6
4	1.0	0.18
5	0.75	0.2
6	1.0	0.41

Relative concentrations expressed as the GMT dilution end-point titre of the preparation divided by the GMT dilution end-point titre of the vCJD preparation measured in the same laboratory. See Table 2B for an alternative analysis based on individual Western blot runs as opposed to GMT results.

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