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Report

Working Group on International Reference Materials for Diagnosis and Study of Transmissible Spongiform Encephalopathies (TSEs):

Second Meeting

Geneva, Switzerland
25-26 May 2000

WORLD HEALTH ORGANIZATION
Blood Safety and Clinical Technology
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WHO Working Group on International Reference Materials for Diagnosis and Study of Transmissible Spongiform Encephalopathies (TSEs)
2nd Meeting

WHO Headquarters, Geneva
25 - 26 May 2000

Report

1. INTRODUCTION

The Second Meeting of the WHO Working Group on International Reference Materials for Diagnosis and Study of Transmissible Spongiform Encephalopathies (WHO TSE Working Group) was held at WHO in Geneva on 25-26 May 2000. The meeting was opened by Dr Yasuhiro Suzuki, Executive Director of the Health Technology and Pharmaceuticals Cluster, WHO. Dr Suzuki emphasized the importance of establishing a WHO Repository of internationally agreed-upon reference materials to facilitate development of methods for diagnosis and better understanding of the TSE diseases, as well as for the validation of the ability of pharmaceutical procedures to remove the agents. The establishment of the reference materials would also be essential for regulatory decisions in the pharmaceutical and biologicals field.

2. HUMAN TSE BRAIN-DERIVED MATERIALS: MASTER PROTOCOL FOR A WHO COLLABORATIVE STUDY

At the previous meeting, the WHO TSE Working Group recommended that candidate biological reference materials for human TSEs be prepared from brains of cases of sporadic and variant CJDs and from a similarly processed uninfected human brain. Responding to this suggestion, four brains were provided by the National CJD Surveillance Unit, Edinburgh, and homogenized by the Institute of Animal Health, Compton, UK. The four preparations of human brain homogenate were aliquotted and will now be distributed by NIBSC to participants in the WHO collaborative study. The preparations were from one uninfected brain, one brain from a case of variant CJD (vCJD) and two cases of sporadic CJD (sCJD). All four brains were from individuals homozygous for the allele encoding methionine at codon 129 of the prion-protein gene (PRNP).

These materials are presented as 10% homogenates in 0.25M sucrose in pyrogen-free water. All specimens have been stored at -80°. The materials will be assayed by immunoblot and other *in vitro* and *in vivo* methods. A master protocol describing the preparation of the brains and instructions for participants is attached as an Annex.

After conclusion of the collaborative study, WHO intends to offer these reference materials as calibrants to laboratories attempting to optimize a variety of *in vitro* and *in vivo* diagnostic procedures for TSEs. They will also be used later to establish WHO proficiency panels of serially diluted, randomized and coded samples.

2.1. Immunoassays

The WHO TSE Working Group recommended testing of the candidate biological reference materials by a variety of available immunoassays for PrP^{Sc}. Samples of the materials will be sent out unblinded and undiluted for PrP^{Sc} detection according to the immunoassay methods used in the participating laboratories. Results of immunoassays should be available for consideration by the Working Group at its next meeting.

2.2. Infectivity assays

A variety of transgenic-mouse models is available including PrP-knockout mice carrying different alleles of the human PRNP gene, chimeric human-mouse gene or the bovine PrP gene. Transgenic animals carrying the PRNP gene are susceptible to sCJD agents, and serial passage in the same transgenic line does not further reduce incubation periods. However, these transgenic lines are less susceptible to vCJD than are some lines of wild-type mice. On the other hand, transgenic mice carrying the bovine PrP gene are more susceptible to vCJD than are wild-type mice, but serial passage of vCJD agent further reduces the incubation period in this transgenic line.

The WHO TSE Working Group recommended that titrations of human CJD reference materials be conducted in a variety of susceptible animals. As described for the *in vitro* studies, the *in vivo* studies will also be conducted according to in-house protocols of participating institutions. The time required to obtain conclusive results will be at least two years.

3. TERMINOLOGY FOR TYPES AND SUBTYPES OF HUMAN PRP^{SC}

Identification of clinical and pathological subtypes may be important for improving the diagnosis and epidemiological surveillance of TSEs. Subtyping based at least partly on differences in glycoforms of PrP^{Sc} has been proposed. The Working Group reviewed information on subtypes of human PrP^{Sc} based on biochemical properties, including relative abundance of the different glycoforms and the size of the unglycosylated protease-resistant fragment.

Two different proposed terminologies have many areas of agreement, and the proponents of each terminology agreed to provide representative samples to the WHO for distribution to participants in laboratories interested in resolving discrepancies. The Working Group agreed that a provisional harmonized protocol for PrP^{Sc} terminology would be elaborated by Professor Herbert Budka in consultation with Professors John Collinge and Pierluigi Gambetti within three months. Samples of CJD brains

containing various forms of PrP^{Sc} will be identified and prepared by Professors Collinge and Gambetti and sent to NIBSC for coding and distribution to ten to fifteen interested laboratories. Detailed laboratory Western Blot protocols from both laboratories will also be distributed to participating laboratories. Participants will be invited to take part in the study by Professor Budka, who will report on progress at the next meeting of the WHO TSE Working Group.

4. ANIMAL TSE BRAIN-DERIVED MATERIALS

Studies similar to that for the human CJD brain material would be useful for the animal TSEs as well. Professor Gerhard Hunsmann outlined the EU project on titration of BSE in primates by intracerebral and oral routes. The currently available supply of BSE-infected material from UK is only sufficient to carry out the transmission studies. The Working Group requested that as much extra material as possible be collected and added to the pool of starting material so that some can be set aside to provide a well titrated BSE reference material.

Reference materials for animal TSEs, particularly BSE, will be of great value to both WHO and to the Office International des Epizooties (OIE), represented at the meeting by Mr Gerald Wells, of the Ministry of Agriculture, Fisheries and Food, UK. The WHO TSE Working Group offered to collaborate with OIE and to help develop protocols and materials analogous to the WHO candidate Reference preparations. There is particular interest in obtaining BSE materials that have already been titrated in cows.

5. HUMAN TSE BLOOD-DERIVED REFERENCE MATERIALS

The issue of suitable reference materials to facilitate the study of blood from humans and animals with TSEs is complex. It is known that infectivity can be detected consistently in the blood of rodents with experimental TSEs, whereas it has never been convincingly demonstrated in the blood of sheep, goats, cows or humans. Infectivity is found in plasma, buffy coat and erythrocytes of rodents but only at low titres, typically about 10 infectious unit per ml of whole blood. Because the infectivity titres are low, very large volumes of blood would be required for useful reference preparations. The frozen storage of any blood components other than plasma is problematic and, although separated lymphocytes can be frozen in an appropriate matrix, that procedure may reduce the infectivity or the amount of PrP^{Sc}.

A subgroup composed of Drs John Collinge, Frank van Engelenburg, Antonio Giulivi, James Hope, Robert Rohwer, and Jiri Safar, coordinated by Dr James Ironside, will exchange and harmonize protocols for obtaining and treating blood for eventual use as reference materials. Irrespective of their possible infectivity, samples of blood from patients with CJD are needed to test future TSE diagnostics; protocols for collection, treatment and storage of blood for that purpose should also be harmonized and will be considered by the above subgroup.

The WHO TSE Working Group concluded that, at this stage, a low-titer brain spike for preparing infected blood to assess putative diagnostic tests would be a useful first step. The form of spiking materials (e.g., microsomal fractions or other

preparations) will be considered at the next meeting. Dr Rohwer will prepare an analysis of possible spiking materials. Standard low-titre spikes based on the candidate CJD biological reference materials will be possible only after the potency of the human brain-derived preparations has been elucidated.

6. CALENDAR OF ACTIVITIES

The next meeting of the WHO TSE Working Group will be scheduled in about eight months when results of immunoassays and attempts to harmonize PrP^{Sc} terminology should be available. A tentative date at the end of January or early February 2001 seems to be realistic.

ANNEX 1

WHO Collaborative Study for Characterization of Preparations as References for Homogenates of Brain Samples from CJD cases

Objectives

To assess the suitability of preparations as references for use in the detection of infectious material in homogenates of brain samples from cases of CJD by assaying for:

1. PrP^{sc} by immunoblot.
2. PrP^{sc} by other *in vitro* methods.
3. PrP^{sc} by *in vivo* methods.

Materials

Four preparations of human brain homogenate will be analysed. All are homozygous for methionine at codon 129 of the prion gene, and are presented as 10% homogenates in 0.25M sucrose in pyrogen free water. One is from an uninfected brain, one from a case of vCJD and the other two from cases of sporadic CJD. The bioburden on blood agar plates ranged from 200 to 600 cfu per ml at the time of preparation and all specimens have been stored at -80° since. Further details of the materials and their preparation are given in the appendix. As the materials have been stored in the frozen state it is likely that they will be aggregated on thawing.

Caution

These preparations are not for administration to humans. The preparations are homogenates of human brains suffering from CJD and should be regarded as hazardous to health. They should be used and discarded according to your own national and laboratory safety procedures.

1. Immunoblot assay for PrP^{sc}

Design of the Study

Participants will be sent 4 vials of each material. Each vial contains a nominal 0.5ml of homogenate. All samples should be stored at -70°C or less on receipt. Participants are requested to perform four independent immunoblot assays for PrP^{sc} on each of the four materials, preferably one week apart using the method in use in the laboratory. A fresh vial should be used for each independent assay, and dilutions of all four materials should be tested at the same time to allow the PrP^{sc} content of each preparation to be determined in the same run.

For the first assay of the four materials, participants should assay ten-fold dilutions of the preparations in order to determine the end-point of each preparation. Five ten-fold dilutions (10^{-1} to 10^{-5}) should be adequate to determine the end-points of the preparations. In the remaining three assays, five half log dilutions (i.e. 1:3.3) around the end point should be assayed.

Results

Data sheets are provided so that all relevant information can be recorded. A separate data sheet should be completed for each assay. Scans and/or photographs should be included. A method form should be completed for each investigator.

Method form WHO-CJD Collaborative Study - 1. Immunoblot

Investigator: Institute:

Method of dispersion of sample on thawing

Dilution matrix

Sample treatment

Volume loaded

Gel composition

Detector antibody

Method of detection

Definition of end point
Other

2. *In vitro* assay for PrP^{sc}

Design of the Study

Participants will be sent 4 vials of each material. Each vial contains a nominal 0.5ml of homogenate. All samples should be stored at -70°C or less on receipt. Participants are requested to perform four independent *in vitro* immunoassays for PrP^{sc} on each of the four materials, preferably one week apart using the method in use in the laboratory. A fresh vial should be used for each independent assay, and dilutions of all four materials should be tested at the same time in one run to allow the PrP^{sc} content of each preparation to be determined in the same run.

For the first assay of the four materials, participants should assay ten-fold dilutions of the preparations in order to determine the end-point of each preparation. The dilutions required depend on the predicted sensitivity of the assay. In the remaining three assays, half log dilutions (i.e. 1:3; 3 dilutions) on either side of the end point should be assayed.

Results

Data sheets are provided so that all relevant information can be recorded. A separate data sheet should be completed for each assay. A method form should be completed for each investigator.

Method form - WHO-CJD Collaborative Study - 2. *in vitro* methods (non-immunoblot)

Investigator: Institute:

Brief description of method including method for dispersion of the sample:

3. *In vivo* assays for infectivity

Design of the Study

Participants will be sent 2 vials of each material which will be identified as normal, vCJD or sCJD. All samples should be stored at -70°C or less on receipt. Participants are requested to perform a quantitative infectivity assay using the design and strain or strains of animals, normal or transgenic in use in the laboratory.

Results

Data sheets are provided so that all relevant information can be recorded. A separate data sheet should be completed for each assay. A method form should be completed for each investigator.

Method form - WHO-CJD Collaborative Study

3. *in vivo* methods

Investigator: Institute:

Animal

Site of inoculation

Age at inoculation

Number of animals per dilution

Dilution matrix

Volume inoculated

End point determination

Histopathological confirmation

Calculation of titre (e.g. incubation period or end point)

Other

CJD Collaborative Study

Name:

Address:

Tel:

Fax:

I would like to participate in the CJD collaborative study on:

	Yes	No
1. Immunoblot assay		
2. Other *in vitro* diagnostics		
3. *in vivo* assays		

The candidate CJD infectivity reference panel members are positive for CJD infectivity. This material will be shipped as an infectious agent. Do you require an import permit or to have special documentation attached to the package?

Yes/No* If yes, please attach.

(* delete as appropriate)

Brief description of method(s) to be used:

I understand that the materials to be distributed in this collaborative study must be regarded as infectious and I accept full responsibility for the use and disposal of the materials which I receive.

Signed:

Date:

Please return to Dr P Minor, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Herts, EN6 3QG, UK

Fax: + (44) 1707 646730

APPENDIX

Preparation of materials

In all cases the brain samples are portions of grey matter enriched frontal cortex. The disease was histologically confirmed in the cases of CJD, and samples of brains from the cases of CJD have been shown to transmit infection to susceptible mice and to be of acceptable toxicity. The clinical details of the cases are in the possession of Dr Ironside of the CJD Surveillance Unit, Edinburgh.

Homogenates were prepared and ampouled at the Institute of Animal Health, Compton, using a new homogeniser bit for each brain and subsequently stored at NIBSC in a dedicated freezer at -86°C. All materials were stored and transported in dry ice or at -86°C.

Preparation of Homogenates at IAH

Equipment:

Eppendorf EDOS 5222 Liquid Dispensing System (repeatability of delivery ± 0.3%)
Camlab Omni - Mixer ES Homogeniser
Class 2 Microbiological Safety Cabinet. (F8C, Stewart Building, IAH, Compton)
Primary container - Nalgene sterile cryogenic vial 1.2ml (Polypropylene; high density polyethylene closure. Cat No. 5000-0012).
Secondary container - Nalgene 9x9 white polycarbonate cryobox (Cat No 5026-0909)

Solution:

0.25M Sucrose (MW 342.30): 85.56g/1 in pyrogen-free water. Pre-chilled to ~4°C.

Procedure:

a) Weigh frozen brain. Thaw sufficiently to allow slicing into 4-5g pieces and transfer to homogenisation container.
b) Homogenise approximately 20g and 108ml sucrose solution in sealed 250ml container using programmable Omni-Mixer set at 4000rpm, 6 x 30 seconds.
c) Transfer homogenate to 2l beaker on ice/salt. Stir on magnetic stirrer throughout.
d) Repeat homogenisation: 4 x 20g brain to create a pool of 1l of 10% brain homogenate.
e) Dispense 100 x 0.5ml into pre-labelled, Nalgene 1.2ml cryovials, place in 9 x 9 Nalgene cryoboxes and immerse in bleach (10% Chloros) for 15 minutes at 4°C. Repeat to dispense remaining homogenate.
f) Rinse cryoboxes in water to remove surface bleach. 'Flash freeze' by immersing in liquid nitrogen for 5 minutes.
g) Transfer onto dry ice for transportation to the storage facility at NIBSC (-86°C)

ANNEX 2

WHO WORKING GROUP ON INTERNATIONAL REFERENCE MATERIALS FOR DIAGNOSIS AND STUDY OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES (TSEs)
Second Meeting: 25-26 May 2000
WHO Headquarters, Geneva

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