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Report

WHO Consultation on Diagnostic Procedures for Transmissible Spongiform Encephalopathies: Need for Reference Reagents and Reference Panels

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REPORT

OPENING REMARKS

Transmissible spongiform encephalopathies (TSEs) include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) and scrapie of sheep and goats. The appearance of new-variant CJD, a disease likely to have resulted from human infection with the BSE agent, increased the awareness that these diseases pose a risk to public health. Many urgent questions have been raised about the safety of animal-derived products and by-products entering the food chain or used in medicine, and public health policies worldwide have already been affected.

A WHO Consultation was convened in March 1997 (1) to address implications of human and animal TSEs for the safety of medicinal products and medical devices. Since that time there have been important developments with potential application to improved diagnosis of TSEs and detection of the TSE agents. There have also been changes in national public health policies and a growing commitment to improved control of the diseases. It is now appropriate to consider additional international activities to advance these efforts.

The accurate pre-mortem diagnosis of TSEs is difficult and reliable diagnostic methods using easily accessible samples, such as human blood or serum, are urgently required. Although novel diagnostic procedures for TSEs have recently been described, they are still at a relatively early stage of development. The need for global harmonization and for a Panel of International Reference Materials is becoming clear.

A Consultation of scientists active in relevant aspects of TSE research was convened at WHO in Geneva on 22-23 March 1999. The Consultation was opened by Dr Michael Scholtz, Executive Director of the Health Technology and Pharmaceuticals Cluster, WHO. Dr Scholtz stated that the objectives of the Consultation were to evaluate progress so far, to assess needs for standardization, and to plan further WHO activities for development of appropriate reference materials and reagents. Improved diagnosis should help to reduce the risk of transmitting disease to animals and to human populations through exposure to contaminated food or to biological materials used in the production of medicinal products. Reference preparations will be an essential tool in the validation of diagnostic procedures and their application to many aspects of health technology and pharmaceuticals. Decisions concerning the safety of pharmaceutical and blood products will be affected by these developments.

Dr P. Minor (United Kingdom) was chairperson of the consultation and Dr M. Pocchiari (Italy) and Dr D. Asher (USA) were nominated rapporteurs. A list of participants is attached as Annex 2.

1. BACKGROUND ON TSEs

TSEs or prion diseases are fatal neurodegenerative disorders that affect both humans and animals. By the time of death, subjects with TSEs usually show some degree of spongiform degeneration in the brain with accumulation of an abnormal protease-resistant form (PrP^{Sc} or PrP^{res}) of the normal protease-sensitive cellular prion protein (PrP^{C} or PrP^{sen}). It is debated whether the agent causing TSEs is PrP^{Sc} alone,

whether there is an additional "informational" component present besides PrP^{Sc}, or if some unknown exogenous agent induces the abnormal conformation of the protein. If PrP^{Sc} is to be used as a marker for infectivity, it is essential to determine whether it is always associated with infectivity in different tissues or whether an infective component can be separated from PrP^{Sc}. Studies transmitting infection are time-consuming and expensive, but they are obligatory to verify the presence of a transmissible agent and to define strains of agent. Analyses of the relative molecular mass (Mr) and glycoform ratio of the protease-resistant PrP^{Sc} fragments have been proposed as an alternative or adjunct to traditional strain-typing techniques, and validation of this approach is ongoing.

CJD is the most common form of human TSE, occurring sporadically in about 85% of cases. Most of the other 15% of CJD cases are associated with mutations in the gene encoding PrP (the *PRNP* gene). However, many CJD patients with *PRNP* mutations have no recognized family history of CJD, and gene analysis must be performed to identify them. Other hereditary forms of human TSE are Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI). There is still no consensus on whether *PRNP* mutations alone are sufficient to cause TSE or increase susceptibility to an exogenous agent. Kuru is an almost extinct disease transmitted by ritualistic cannibalism among Fore-speaking people in Papua New Guinea. Accidental infections with TSE agents have also occurred in Western countries as a consequence of iatrogenic exposures.

In 1996, a clinicopathological variant of CJD ("new-variant CJD," abbreviated nvCJD or vCJD), affecting unusually young individuals, was recognized in the UK. It is most plausible that nvCJD occurs in people who were exposed to BSE-infected material, probably through contaminated food. As of 22 March 1999, there had been 39 definite and probable cases of nvCJD identified in the UK and one case in France.

The diagnosis of CJD encompasses a range of overlapping criteria, none of which is sufficient alone. Clinical criteria have been developed and partially validated for the diagnosis of CJD in life, although the gold standard for diagnosis is the neuropathological examination of brain tissue, now augmented by immunodetection of PrP^{Sc}. The identification of PrP^{Sc} by immunostaining in lymphoid tissue of nvCJD patients (and not in those tissues of classical CJD patients) raised the possibility that blood, blood components and plasma-derived medicinal products might contain sufficient infectivity to transmit disease from human to human. The implications of these findings, together with the fear that medicinal products or medical devices derived from animals potentially incubating BSE might constitute a vehicle to transmit BSE to humans, prompted scientists from research and industrial laboratories to search for more sensitive, rapid and economical diagnostic tests to detect TSE infections and to identify materials contaminated with TSE agents.

2. INFECTIVITY ASSAYS

Experimental transmission to laboratory animals is the only established method for the identification and quantitation of infectivity and for the isolation and characterization of different strains of agent. The level of infectivity in a sample can be estimated either by end-point titration or by incubation period. Transmission of disease to an animal of the same species yields the maximum efficiency of infection, whereas interspecies transmission is hampered by a "species-barrier" effect, not predictable but often substantial, which reduces efficiency of infection, thus diminishing the sensitivity

of the assay and increasing incubation periods in animals successfully infected. The existence of multiple strains of scrapie and CJD agents with a variety of properties in different host animals further complicates selection of suitable animal models (2).

2.1 Conventional animal models

Non-transgenic models have been extensively used to show the transmissibility of naturally occurring human and animal TSEs. Natural scrapie has been regularly transmitted from infected sheep or goats to mice. During the last 40 years, over 20 strains of scrapie have been identified and characterized using a panel of genetically defined mouse lines. Nonhuman primates are currently the species most susceptible to experimental infection with human TSE agents. However, even among primate species with similar or identical PrP-gene sequences there appear to be differences in susceptibility to human TSE agents. For ethical and logistic reasons, several laboratories have also attempted, and sometimes succeeded, to transmit human TSEs to rodents.

The most sensitive method to detect infectivity in human TSEs may involve inoculation into primates followed by prolonged observation, but this is not often practical. Selection of the most suitable animal model depends on the purpose for which it is intended and the best animal model may not be obvious; for example, when infected with nvCJD brain homogenate, non-transgenic RIII mice give higher transmission rates (3) than do some lines of transgenic mice carrying the *PRNP* gene. Thus, the use of animal models to study pathogenesis of TSEs may be misleading unless all models give similar results and comparability of results from different laboratories is not always clear. Studies in primates and ruminants, albeit expensive and logistically difficult, remain important to confirm the relevance of experiments in more accessible laboratory rodents and to estimate the relative sensitivity of assays in rodents.

2.2 Transgenic models

Transgenic (Tg) mice overexpressing foreign PrP genes have been developed since 1989. Since then, a large number of Tg-mouse lines have been engineered bearing different PrP constructs from various TSE-susceptible species (human, sheep, bovine, etc.). In general, achieving efficient transmission with short incubation periods has required either ablation of the endogenous mouse-PrP gene in animals bearing the transgene, the introduction of a chimeric PrP construct (e.g., mouse-human), expression of multiple copies of the transgene, or a combination of those. However, it may not be valid simply to equate a shorter incubation period with greater sensitivity. There is need to correlate the infectivity titres of standard samples between non-Tg and Tg models. Promoter genes appear to affect the incubation periods of scrapie in Tg mice, and the location and number of transgenes inserted may also play a role. These factors remain to be elucidated better. It remains unclear which features are most relevant to the goal of deriving assay animals able to detect infectivity across a species barrier with high sensitivity.

A Tg-mouse line expressing multiple copies of the bovine PrP gene, introduced in a large cosmid to reduce genomic positional effect, was highly susceptible to infection with unpassaged preparations of nvCJD, BSE and scrapie agents (4), all of which had similar short incubation periods, less than 250 days. As they do in RIII mice, BSE and nvCJD agents behaved in those Tg mice as the same strain with localization of PrP^{Sc} and amyloid plaques similar to that in the original diseases. The bovinized mice

had similar incubation periods when infected with either BSE prions from bovine brains or from mouse-passaged agent, suggesting that there was no species barrier between cow and Tg mouse.

Other Tg mice have been produced (5) by homologous replacement, leaving the introduced gene under control of its natural promoter in its natural location. Such mice might be expected to provide a more relevant representation of natural infections and to be especially useful for experimental studies of pathogenesis. These animals have been used to demonstrate the identity between the scrapie incubation-period gene (sinc) and the PrP gene.

Tg mice have recently been used to investigate potential therapeutic compounds. A critical event in the pathogenesis of TSE is the conversion of normal cellular PrP (PrP^{C}) to the abnormal TSE-associated PrP^{Sc} form. Compounds that inhibit that transformation would be strong candidates as prophylactic or even therapeutic drugs. A fast model of hamster scrapie in "hamsterized" Tg mice is being used to explore the use of inhibitors of PrP^{Sc} formation as potential chemotherapeutics (6). Tg mice have also been extensively used to study involvement of B cells (7), T cells and other components of the immune system in the pathogenesis of experimental scrapie in mice.

3. DEVELOPMENTS IN DISEASE-SPECIFIC PrP-BASED ASSAYS

The hallmark of TSEs is the accumulation of PrP^{Sc} in the brain, and, to a lesser extent, in other tissues. The detection of PrP^{Sc} in the cerebral tissue of affected individuals by immunochemical techniques is rapid, sensitive, and provides highly reliable results. These methods are much less time-consuming than infectivity assays and can be performed either in fixed tissue (immunohistochemistry) or unfixed tissue (immunoblotting [IB], ELISA, DELFIA, conformation-dependent immunoassay). The amount of PrP^{Sc} in a given tissue depends on the host species, the strain of the infectious agent, the route of infection and the time elapsed after infection. PrP^{Sc} immunoassays should be interpreted with special caution when kinetic curves of PrP^{Sc} accumulation in a specific tissue are not available.

In the preclinical stages of infection, PrP^{Sc} may be undetectable in the brain, but it may be detected in lymphoid tissues. Improvement in the sensitivity of these assays is fundamental to detect low levels of PrP^{Sc} at the preclinical stages of the infection, for the screening of PrP^{Sc} in tissues and fluids outside the central nervous system (CNS), and in the evaluation of bulk or final biological products.

A critical point for the standardization of these techniques is to identify and make available a reference panel of antigens (either purified PrP^{Sc} or recombinant PrP from different species or both) and of anti-PrP antibodies. Spiking normal biological materials with TSE-infected brain tissue or with extracted PrP^{Sc}, while useful and relatively easy to control, may not accurately reflect the properties of TSE agents in naturally infected biological source materials. More biologically relevant reference materials should also be considered.

3.1 Immunohistochemistry

Immunohistochemistry consists of the microscopic detection of immunostained PrP^{Sc}. This technique not only detects PrP^{Sc} but also reveals its pattern and topographical distribution in the tissue. Critical to the sensitivity and the specificity of

immunohistochemistry are the tissue-fixation protocol and additional procedures employed to unmask PrP^{Sc} antigenic sites and to eliminate normal PrP^{C}. Studies of sheep with clinical signs of scrapie and in humans with TSEs showed that immunohistochemistry had a higher sensitivity than did classical histopathology. This finding may have a critical importance to select adequate diagnostic protocols. In scrapie-infected sheep, PrP^{Sc} was consistently detected in lymphoid tissues early in the incubation period (8), before clinical disease developed. This may prove to be true for nvCJD as well.

Immunohistochemical techniques for detecting PrP^{Sc} in formalin-fixed human tissues have been carefully optimized, yielding highly reproducible results. A simple new protocol, fixing brain tissue in Carnoy fixative (9) instead of formalin and then treating sections with proteinase K and guanidine thiocyanate before immunostaining, was described. This method yielded a strong and very consistent labeling of all types of PrP^{Sc} deposits and preserved tissue morphology exceptionally well. Most important, it permitted subsequent efficient extraction of PrP^{Sc} from paraffin-embedded material for IB or other methods of analysis usually performed with unfixed tissues.

Since many investigators have a strong expertise in TSE immunohistochemistry and have published several comparisons of protocols, the standardization of these techniques should not be a difficult task. The establishment of standard protocols (10) should include the selection and the supply of reference positive and negative control sections and of anti-PrP-specific antibodies. Collections of human and animal tissues infected with TSE agents are already available from several sources; some of the specimens have been titrated for infectivity.

3.2 Immunoblot

The identification of PrP^{Sc} by IB is a highly specific test requiring either unfixed or Carnoy-fixed tissue. PrP^{Sc} is comprised of distinct types according to the Mr of its protease resistant core fragment following removal of its two sugar chains. Protease-resistant (PrP^{Sc}) fragments PrP (27-30) may be further subclassified based on the ratio of the three glycoforms (di-, mono- and non-glycosylated PrP) which commonly migrate as three electrophoretic bands with different molecular weights and relative abundances (11). These parameters characterize the so-called "PrP^{Sc} typing" and may provide useful information on the putative strain of the TSE agent. At present, there is no universally accepted classification for human PrP subtypes as detected by Western blot, a matter demanding urgent resolution.

A sensitive IB based on the monoclonal antibody 6H4, which recognizes human, bovine, sheep, pig, mouse and hamster PrP, has been developed (12). This test detected PrP^{Sc} in bovine cerebral tissue soon after slaughter. A sensitivity of 97% was estimated under adverse field conditions.

Another IB assay (13), recently developed, was useful to monitor the distribution of PrP^{Sc} in fractions obtained from scaled down simulations of plasma-derived medicinal products. This study showed that TSE infectivity partitioned into different plasma fractions and generally tracked with PrP^{Sc}.

A panel of monoclonal antibodies (14) that recognize different regions of the PrP molecule in a variety of mammals has also been generated. These monoclonal

antibodies are useful to demonstrate different PrP^{Sc} fragments and may further refine PrP^{Sc} typing .

In another study, amounts of infectivity and PrP^{Sc}, estimated using the 3F4 monoclonal antibody (15) were compared in an experimental model of scrapie in hamster. The analysis suggested that the measurement of PrP^{Sc} in CNS tissue may be used to estimate the level of infectivity, but the sensitivity of assays for PrP^{Sc} must be very high if it is to be useful in detecting small amounts of infectivity.

3.3 ELISA, DELFIA, and Conformation-dependent Immunoassay

ELISA and DELFIA (Dissociation Enhanced Lanthanide Fluoro Immuno Assay) immunoassays bind PrP to multi-well plastic plates and then probe with specific antibodies. The detection of the protein is based either on an enzyme-catalyzed colorimetric reaction (ELISA) or on time-resolved fluorometric measurement of a lanthanide chelate linked either to the primary detecting antibody or to a secondary antibody (DELFIA). The "two-site" DELFIA method (16) has provided reproducible and sensitive measurements of PrP in blood and blood fractions and may be developed to form the basis of a diagnostic test for TSEs. DELFIA is sensitive to the state of aggregation and conformation of PrP and has already been validated as a confirmatory test for scrapie in hamsters.

A conformation-dependent immunoassay (CDI) has been developed to discriminate between PrP^{Sc} and PrP^{C} (17). CDI identifies PrP^{Sc} in samples without prior proteinase-K digestion and quantifies the PrP isoforms by following the binding of antibody to denatured and native forms of the protein simultaneously. CDI was reported to detect less than 1 ng/ml of PrP^{Sc} in homogenates of brain from scrapie-infected hamsters in the presence of a 3-thousand-fold excess of PrP^{C} with a dynamic range of five orders of magnitude. The method also revealed differences in the ratios of denatured to native PrP that might serve to define strains of TSE agents prion and to indicate origins of infection. CDI has been proposed as a potentially reliable method to detect very low levels of infectivity.

3.4 Capillary Immunoelectrophoresis

The capillary immunoelectrophoresis (CIE) assay for PrP is based on competition between PrP and fluorescein-labeled synthetic PrP in their binding to antibodies raised against the peptides (18). Thus, when PrP is present in the assay mixture, it competes with the fluorescent labeled peptide for binding to the antibody. Fluorescence is detected by a laser-induced fluorescence detector. About 50 attomoles of peptide, equivalent to ~1.5 picograms of PrP, can be detected in the capillary, so that concentrations of PrP in the nanomolar range can be measured. Normal and abnormal forms of PrP are distinguished by digesting one of a pair of replicate samples with proteinase K before extraction and then testing both samples. This technique detected PrP^{Sc} in extracts of buffy coats from the blood of sheep infected with scrapie and of mule deer and elk infected with chronic wasting disease before the onset of clinical illness. Similar samples prepared from buffy coats of normal animals did not contain the protease-resistant PrP^{Sc} as measured by this assay. Animals positive for buffy-coat PrP^{Sc} by CIE later became ill, died and were confirmed by conventional tests to have a TSE. CIE may provide an assay suitable for pre-clinical diagnosis of TSEs and for detection of PrP^{Sc} in biological and pharmaceutical products. The potential utility of this assay for pre-morbid diagnosis of human TSEs is apparent. However, the

sensitivity and specificity of CIE as an assay for TSE agents in specimens of human-derived materials remain to be determined.

4. DEVELOPMENT OF NON-PrP-BASED ASSAYS

The definitive diagnosis of TSEs is usually based on the neuropathological examination and the identification of PrP^{Sc} in post-mortem tissue samples. Cerebral biopsy is not indicated if there is no potentially treatable disease in the differential diagnosis, since the procedure may worsen a patient's condition; more accessible diagnostic markers would be helpful. Abnormal levels of several cerebral proteins (NSE, S-100, tau, 14-3-3) have been frequently observed in the cerebrospinal fluids (CSF) of CJD-affected patients and, less commonly, in CSF of those with other neurological diseases. The identification of 14-3-3 protein by Western blotting (19) appears to be the most sensitive available test for CJD, and, though not specific, a positive test supports the diagnosis of CJD in an appropriate clinical context.

5. CONCLUSIONS AND RECOMMENDATIONS

Reliable non-invasive tests to detect asymptomatic subjects during the long incubation periods of CJD are required. Such tests could be used to exclude potentially infectious donated blood, organs and tissues as well as for a variety of epidemiological studies. Most important, such tests would identify people who would benefit from therapeutic interventions aimed at eliminating infection, or at least delaying the onset of disease. Accurate pre-mortem testing would also improve control of animal TSEs.

In view of the serious implications of TSEs for human medical products and the rapid developments in the field, especially in regard to blood safety, the Consultation suggested several actions to facilitate development of improved diagnostic tests based on research methods currently available.

5.1 WHO Working Group

A WHO Working Group on International Reference Materials for Diagnosis and Study of TSEs should be formed as a matter of urgency.

5.2 Neuropathology

(a) Histopathological slides and materials should continue to be exchanged and attempts made to harmonize interpretation.

(b) There is a need to standardize procedures and reagents used in neuropathology as much as possible.

(c) There is also a need to define PrPSc typing in order to clarify the classification and nomenclature of human TSE cases. Collaborative studies to this end are encouraged.

5.3 Reference materials

(a) There is a need to develop reference materials to compare available assay systems.

(b) Positive and control material derived from brain and blood of humans and animals with TSEs should be identified. This would include material from BSE, sporadic and nvCJD brains and material derived from the same sources passaged in Tg mice as well as titred samples of established laboratory strains of TSEs. Such references would be valuable in attempts to harmonize the results obtained with various models of primary transmission and to compare various diagnostic approaches. Other tissues and animals may become significant with time.

(c) Collection of samples of a range of tissues from preclinical animals such as sheep incubating scrapie, cows incubating BSE and animals experimentally infected with CJD or other TSEs will also be needed.

5.4 Reagent banks

The need for reagent banks containing monoclonal antibodies and genetically engineered PrP should be considered. Distribution of specific mouse strains and TG mouse lines should also be encouraged.

5.5 Ethical issues

All studies involving human subjects, living or dead, should be conducted in accordance with national ethical standards including respect for confidentiality and privacy.

ANNEX 1

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ANNEX 2

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WHO Consultation on Diagnostic Procedures for Transmissible Spongiform Encephalopathies: Need for Reference Reagents and Reference Panels Geneva, Switzerland, 22-23 March 1999

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