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Human Medicines Evaluation Unit

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EMEA WORKSHOP ON APPLICATION TO PHARMACEUTICALS OF ASSAYS FOR MARKERS OF TSE

Held on 19-20 January 1999

REPORT TO THE CPMP FROM THE BIOTECHNOLOGY WORKING PARTY

SUMMARY

In early 1998, the CPMP released a position statement on new variant Creutzfeldt-Jakob disease (nvCJD), CPMP/201/98. This statement included comment on the desirability of developing assays for markers of transmissible spongiform encephalopathy (TSE). A follow up meeting was held at the EMEA to share information on the current status of these assays and their possible use as diagnostic or screening tools and in evaluating manufacturing processes for pharmaceuticals.

The conclusion drawn from the meeting is that there has been substantial progress in the development of *in-vitro* and *in-vivo* assays for markers of TSE. However, at present the predictive value of these *in-vitro* assays has not been established, although in experimental models there is some correlation with TSE infectivity. There are a number of technical questions (such as sensitivity, robustness, through-put/automation, establishment of reference materials) which have still to be addressed. These issues have to be resolved before these techniques can be applied on a routine basis to the monitoring of starting materials (animal/human) used in the manufacture of pharmaceuticals. These assays should be considered as an addition rather than as a replacement for other precautionary measures already in place to minimise the risk of transmission of TSE agents. It is likely that one of the first applications of these tests may be as a tool in the evaluation of pharmaceutical manufacturing processes for their capacity to remove/inactivate transmissible agents, and some preliminary work in this area is already on-going. The CPMP position statement on CJD and nvCJD issued in February 1998 and the CPMP 'TSE guideline' remain valid.

I INTRODUCTION

Transmissible spongiform encephalopathies (TSE) are neurodegenerative diseases with common features that occur in both animals and humans. They are associated with spongiform degeneration in the brain and the accumulation of an abnormal form of a naturally occurring glycoprotein, called prion protein (PrP^{c})[1]. The nature of the agents causing TSEs is still unknown. These agents replicate in infected individuals without induction of a detectable immune response. The agents have been reported to be present in certain tissues and fluids of infected humans or animals, which may or may not be clinically ill. Cases of transmission of a human TSE, Creutzfeldt-Jakob disease (CJD), have occurred following treatment with substances of pituitary origin and in recipients of dura mater grafts. Throughout the 1990s the Committee on Proprietary Medicinal Products (CPMP) has, in the light of the available scientific knowledge, considered appropriate measures to minimise the risk of transmission of TSEs via medicinal products derived from human or animal origin, and adopted a precautionary approach.

In 1991 the CPMP published its initial recommendations regarding the possible risk of transmission of TSE via medicinal products and how to minimise it (*CPMP, III/3298/91*). The most recent version

[1] PrP^{sc} is an abnormal isoform of the natural protein PrP^{c}, which is anchored at the surface of many cells in mammals. PrP^{c} and PrP^{sc} have a different resistance towards proteinase K treatment : the endogenous PrP^{c} is completely degraded by proteinase K, whereas PrP^{sc} is partly resistant (giving rise to PrP^{res}).

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was in 1998 (*CPMP/BWP/1230/98*). In early 1998, an EMEA workshop of international experts on TSEs, convened under the auspices of CPMP, considered the emerging information on new variant CJD (nvCJD) and relevant TSEs. In the light of this information, the EMEA published a position statement on nvCJD (in February 1998; *CPMP/201/98*). This statement included comments on the desirability of developing assays for markers of TSE.

Research has been initiated by academia and industry with the aim of developing tests for the detection of markers of TSE. These tests are expected to have the potential to be used by the pharmaceutical industry or their suppliers in various circumstances such as qualification of starting materials, and as a tool in the evaluation of pharmaceutical manufacturing processes for their capacity to remove/inactivate transmissible agents.

At the initiative of the CPMP, a follow up EMEA workshop was held in January 1999 to share information on the current status of these assays and their possible use as diagnostic tools or for screening of starting materials and in evaluating manufacturing processes for pharmaceuticals. Participants included members of CPMP, the Committee for Veterinary Medicinal Products (CVMP) and the Biotechnology Working party, representatives of the European Commission and WHO, as well as experts of the ad hoc working group on TSE agents together with experts from industry and academia.

II CURRENT STATE OF UNDERSTANDING ABOUT TSES – NATURE OF THE AGENT – TISSUE DISTRIBUTION

The current state of understanding about the nature of TSE agents, the pathogenesis of the diseases and tissue distribution of infectivity was summarised in several presentations.

The exact nature of the infective agent is still unknown. It is still debated whether the agent is the prion protein in an abnormal conformation (PrP^{sc}) (*Pruisiner et al., 1998*), whether there is an 'informational' molecular component present in addition to PrP^{sc} (*Hope, 1994; Weissmann, 1991*), or if there is an unknown virus which drives the abnormal conformation of the protein (*Diringer et al., 1994*). While the nature of the agent is unresolved, the question will remain as to whether detection of PrP^{Sc} will always be associated with infectivity or whether an infective component can be separated.

As already indicated in the position statement on nvCJD of February 1998, studies in animals suggest that the lymphoreticular system may be involved in the replication of the agents of TSE, although the extent of involvement may vary between host species and/or agent strains. Further investigations have shown that PrP^{Sc} was detected in all lymphoreticular tissues (tonsils, lymph nodes and spleen) examined from patients with definite nvCJD but was not detectable in tissues from patients with other human TSEs or in controls (*Hill et al., 1999*). This is consistent with the preliminary data available in 1998 that led the CPMP to conclude that there might be an increased level of infectivity associated with the lymphoid system in nvCJD cases compared with other forms of CJD. There is increasing evidence that dendritic cells play an important role in the pathogenesis of TSEs (*Klein et al., 1998; Mabbot et al., 1998*). The precise role of B lymphocytes is still under investigation.

Studies of the distribution of infectivity in blood and plasma fractions of clinically ill mice that had been inoculated intracerebrally with a mouse-adapted strain of human TSE, were published in 1998 (*Brown et al., 1998*). Infectivity was detected at low levels in blood by a bioassay (with intracerebral inoculation), and partitioning into various cellular and plasma compartments was seen. Results of these and other experiments with artificially infected animals are difficult to interpret and their relevance to naturally occurring TSEs is not established. TSE infectivity has not been found in the blood of naturally infected animals, and investigations with sporadic CJD have failed to show any transmissibility by intravenous transfusion *(Scientific Committee on Medicinal Products and Medical Devices, of DGXXIV, 1998*). No new information has become available in the last year that would alter the assessment made by the CPMP in their position statement of February 1998 (*CPMP/201/98*).

As far as the BSE epidemic is concerned, the most recent UK figures provide confirmation of the further decline. There is still, however, no reliable estimation of the eventual number of cases of nvCJD that may occur. As of 31 December 1998, there have been 35 definite and probable cases of nvCJD in the UK (Monthly CJD figures published by Department of Health, UK) and one case in France. There is evidence that the pathological prion protein is detectable, by immunohistochemistry

and western-blot, in lymphoreticular tissues (tonsil/appendix) of nvCJD patients (*Hilton et al., 1998; Hill et al., 1999)*. However, it is still unclear whether this might provide a tool for future estimations of the prevalence of nvCJD (*Ghani et al., 1998*).

On the basis of the latest information on the pathogenesis and infectivity distribution of TSEs, the CPMP position statement of February 1998 is still valid.

III DEVELOPMENT OF ASSAYS – VALIDATION AND PROPOSED UTILISATION

Two categories of assays can be used. The first category is bioassays, which remain the definitive method at the moment, as they are the only way to measure directly infectivity in samples. However, a disadvantage is that they are not rapid. They could be improved, essentially in terms of sensitivity (e.g. species barrier, detection of different strains) and time of response, by the use of transgenic animals (humanised or chimeric mouse; *Prusiner et al., 1998, Collinge et al., 1995, Hill et al.,1997*). Although a lot of progress has been made in this area, further work is needed to evaluate the capacity of such tests and to cross-validate them in relationship to the currently accepted bioassays.

There has been rapid progress in the development of *in vitro* assays in recent years. The proposed tests are based on immune reactions between the antigen (PrP^{res} or PrP^{sc}) and antibodies capable of detecting such antigens. Several strategies have been proposed:

- Western blot, which is a semi-quantitative test, already widely used to confirm the presence of PrP^{res} in tissues of infected animals or patients[2]. They cannot be easily applied as routine tests since they cannot be automated.
- ELISA detection is made through a second antibody that is further detected by an enzymatic reaction[3]
- DELFIA, the detection makes use of a highly sensitive lanthanide fluorescent reaction[4].

In addition, within these three assay systems, tests differ in the type of monoclonal antibody(ies) used for the reactions of capture and detection. Some of the assays take advantage of conformational differences between PrP^{sc} and PrP^{c} for the binding to monoclonal antibodies (*Safar et al., 1998)*. There are also differences in the way samples are prepared and treated during the assay, to improve the sensitivity and specificity of the detection (*Korth et al., 1997*). Particularly, use of proteinase K or precipitation by phosphotungstic acid (*Safar et al.,1998*) have been reported.

The presentations made during the meeting have given an understanding of the state of development of the various methods. However, it became evident that for all the tests presented, it was not possible to propose, at the moment, a validated estimation of their respective performance and to make comparison amongst the tests. The main reason for that is the current lack of standardised preparations. The question of standardised reference preparations is thus a key point in the future development of such tests, as well as in the evaluation and validation of their performances. Collaborative studies should be encouraged as well as availability of standard preparations (strains of prion agents, normal and pathological prion proteins, and monoclonal antibodies). The nature of the material(s) to be used as reference preparation(s) has still to be defined. Material of both human and animal origin should be developed as standard reference preparations to cover the various experimental and routine conditions for use in assays. This needs to be done before they can be proposed as tests in routine use for diagnosis, confirmation or screening. In addition, a correlation

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[2] Only the pathological prion protein PrP^{sc} will result, after proteinase K treatment, in PrP^{res}. The presence of the PrP^{res} signal on the Western blots is thus a measure of the presence of the pathological prion protein in the sample, not of the endogenous PrP^{c}.

[3] A first (capturing) PrP specific antibody, coated on a solid carrier (titer plate) will bind the PrP. A second PrP specific antibody, to which a enzymatic detection system is linked, will bind to the capture antibody-PrP complex. The enzymatic reaction (e.g. colour reaction) is used to measure of the amount of PrP in the sample. In most systems presented, the sample has to be pretreated by proteinase K to differentiate PrP^{c} and PrP^{sc}.

[4] DELFIA (Dissociation Enhanced Lanthanide Fluoro-Immuno-Assay) uses the same sandwich principle as the ELISA with a PrP capture antibody and a PrP detection antibody. However, the detection is not enzymatic, but based upon the formation of a fluorescent chelate.

between infectivity and the marker detected by the *in vitro* test method should be a part of the validation of these tests.

For example, at the moment, a test carried out on animal material and found negative would not necessarily mean that the animal, from which the sample was taken, was TSE-free. It could mean that, either the tissue analysed has not been taken at the appropriate time of incubation, and/or that the level of infectivity or PrP^{sc} is too low to be detected. Even when there is very good evidence that an *in vitro* test can reliably detect preclinical and clinical cases, it should still be considered as an addition rather than a replacement for other precautionary measures.

Furthermore, screening for the presence of the agent in human blood is not yet feasible. Indeed, unless infectivity is reliably established in blood of CJD or nvCJD patients, it will be very difficult to evaluate the validity of any assay proposed as a diagnostic or screening tool. In addition, a negative result will never be considered conclusive if no reference standard has been established.

Finally, it is clear that, before any test can be approved and/or recommended by regulatory authorities, they should be carefully evaluated according to relevant criteria such as sensitivity, rates of false negative and false positive results, limit of detection and practicality (e.g. robustness and ease of automation). These criteria still have to be defined.

In the absence of an independent evaluation by authorities, it is not possible, at the moment, to make any recommendation as to the general acceptability of such tests, although some applications can be foreseen and, particularly their use in the evaluation of some manufacturing steps.

IV ASSESSMENT AND EVALUATION OF MANUFACTURING PROCESSES

Several presentations were made on the application of TSE tests in the investigation of removal, inactivation or partitioning of TSE agents by manufacturing processes for biotechnology products and plasma-derived medicinal products. Some results have been obtained using infectivity tests, whereas others are based purely on immunoassay; others use both approaches. Where both tests were used in parallel, a correlation was observed. However, interpretation of all of these results is difficult as it needs to take into consideration the nature of the spiked material (most often brain homogenate) and its relevance to the natural situation, the design of the study (including scaling-down of processes), and the method of detection of the agent, after spiking and after the treatment. Further research is needed to develop an understanding of the most appropriate methodology for validation studies.

In the case of plasma-derived products, the CPMP position statement of 1998 concluded that there is no evidence that sporadic, familial or iatrogenic CJD are transmitted via these products, and that the transmission of nvCJD is very unlikely. It was confirmed during the current meeting that no new evidence had been generated in the last year that shows that the agents are present in human blood (*Scientific Committee on Medicinal Products and Medical Devices, 1998*). If the agent were to be present, it is very likely to be at a very low titre. Nevertheless, the CPMP position statement stated that manufacturers would be encouraged to investigate further precautionary measures that may be applicable in the manufacturing process of plasma-derived medicinal products including the development of methods to remove or inactivate the agent of nvCJD.

Several manufacturers of these plasma-derived products are already investigating the capacity of their processes to remove, partition or inactivate the agents of TSE, and are seeking to identify robust inactivation/removal steps. The results of these studies on alcoholic fractionation processes show a reduction in the infectivity titre in the various fractions through a partition effect (while the infectious agent is not inactivated). These preliminary results also suggest that filtration may be useful for removal. The use of other agents such as detergents and chaotropes, or the combination of such ingredients is also under investigation. When interpreting results it should be born in mind that some procedures are less reliable and robust than others (e.g. partitioning versus removal/inactivation) and that extrapolation from the experiment to the real situation is extremely difficult because of the lack of knowledge of the physical state of the TSE agent naturally in blood if it occurs there at all.

Beyond the particular limitations which apply to TSE validation studies and their interpretation, the major hurdle is identifying steps which will effectively remove or inactivate TSE agents during the manufacture of both plasma-derived and biotech/biological medicinal products. Manufacturers are encouraged to continue their investigations into removal and inactivation methods to identify

steps/processes which would have benefit in assuring the removal or inactivation of TSE agents. If claims are made for the ability of manufacturing processes to inactivate or remove TSE agents, these will have to be substantiated by appropriate validation studies.

V CONCLUSIONS

The meeting was very useful in sharing the most up-to-date information between experts and companies developing assays for the detection of markers of TSE, companies who have applied the assays to the manufacture of pharmaceuticals and representatives of Regulatory Authorities for medicinal products.

The conclusions drawn from the meeting are :

- There has been substantial progress in the development of assays for markers of TSE.
- Companies are developing *in vitro* assays (such as Western blot, ELISA, DELFIA) to detect the pathological prion protein. However, at present the predictive value of these assays has not been established, although in experimental models there is some correlation with TSE infectivity. Bioassays are the only way to directly measure infectivity.
- A number of technical questions (such as sensitivity, robustness, through-put/automation) have still to be addressed. Also, the applicability of these *in vitro* assays to tissues other than neuronal has not yet been clarified. Those issues have to be resolved before these techniques can be applied on a routine basis to the monitoring of starting materials (animal/human) used in the manufacture of pharmaceuticals. It is likely that one of the first applications of these tests may be as a tool in the evaluation of pharmaceutical manufacturing processes for their capacity to remove/inactivate transmissible agents, and some preliminary work in this area is already on-going.
- There is a need for a panel of international reference materials (e.g. materials of human and animal origin). It was stressed that collaborative studies were required to be able to optimise, validate and compare the various immuno-assays under development. These matters will require the involvement of the European Pharmacopoeia.
- Even when tests are available, and when there is very good evidence that the *in vitro* test can reliably detect preclinical and clinical cases of TSE, these tests should still be considered as an addition rather than a replacement for other precautionary measures.
- The CPMP position statement on CJD and nvCJD issued in February 1998 and the CPMP 'TSE guideline' remain valid.

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